THE FATE OF THE INFLAMMATORY MACROPHAGE WITH RESOLUTION OF INFLAMMATION.

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CONTENTS

ABSTRACT

Resolution of inflammation requires the clearance of a large number of inflammatory cells in an ordered manner, such that the tissue architecture returns completely to normal. Neutrophils have been shown to undergo apoptosis and be ingested by inflammatory macrophages (Mø) thus providing a unique method for their clearance that does not induce further pro-inflammatory signals. The fate of the inflammatory Mø however remains unclear. Hence this work investigated the fate of the Mø during the resolution of inflammation.
To test whether the Mø was capable of undergoing apoptosis, human peripheral blood monocytes were isolated and matured into Mø *in vitro*. Using DNA gel electrophoresis to demonstrate the typical "ladder pattern" characteristic of apoptosis and quantifying this by acridine orange fluorescence staining and flow cytometry, we showed that the Mø did not undergo significant constitutive apoptosis (unlike the neutrophil) but cycloheximide could induce apoptosis in these cells. Surface marker changes associated with the induction of apoptosis included the loss of CD14 and a range of cytokine receptors, whilst HLA-DR and Fas were retained far into the apoptotic process. A range of pro and anti-inflammatory mediators (TNF-α, IL-1, IL-4, IL-6, TGF-β, dexamethasone), implicated in induction of apoptosis in other cells, were shown to have little effect on the Mø. Despite this relative resistance to apoptosis, during differentiation into the Mø human monocytes lost the ability to express bcl-2; suggesting either the involvement of a different bcl-2 family member or a non-bcl-2 mechanism protecting the cells from apoptosis.

To study the fate of inflammatory Mø during the resolution of inflammation *in vivo*, a murine model of resolving peritonitis was developed in which the fate of semi-allogeneic adoptively transferred donor inflammatory Mø could be tracked. Red fluorescent labelled donor H-2^{kd} inflammatory Mø were transferred into the peritoneal cavity of recipient H-2^{k} mice which were at the same stage of resolving inflammation. Donor, recipient, and donor cells which had been phagocytosed by recipient Mø, could be distinguished by two colour flow cytometry using a FITC conjugated anti H-2^{d} monoclonal antibody, the phagocytosed cells being red fluorescent labelled but H-2^{d} negative.

Adoptively transferred live inflammatory Mø disappeared steadily from the peritoneal cavity, being undetectable 96 hours post transfer and minimal phagocytosis was evident during this process. In contrast, fixed donor Mø were rapidly phagocytosed by the recipient Mø and these cells could be detected up to a week later. These data suggested that adoptively transferred live inflammatory Mø emigrated from the peritoneal cavity during the resolution phase of inflammation. Labelled cells were detected specifically in the draining lymph nodes and later in small quantities in the liver and spleen but not in a range of other tissues. Thus by contrast with the neutrophil, which meets its fate during the resolution of inflammation locally by undergoing apoptosis and being ingested by inflammatory Mø the inflammatory Mø appears to emigrate from the inflamed site to the local lymph nodes (and possibly the liver and spleen) where it then meets its fate by as yet obscure mechanisms.

ABBREVIATIONS.

ATCC	American Type Culture Collection
BSA	Bovine serum albumin
C5a	Complement fragment 5a
cAMP	Cyclic adenosine monophosphate
CCE	Countercurrent centrifugal elutriation
CSF-1	Colony stimulating factor-1
db-cAMP	Dibutyryladenosine 3'5'-cyclic monophosphate
ECACC	European Collection of Animal and Cell Cultures
EDTA	Ethylene diamine tetra-acetic acid
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-L-methionyl-L-leucyl-phenylalanine
GSH	Reduced glutathione
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GPI	Glycosyl-phosphatidylinositol
HBSS	Hank's balanced salt solution
HBSS w/o	HBSS without calcium and magnesium
ICE	Interleukin-1β converting enzyme
IFN-γ	Interferon-γ
i.p.	Intraperitoneal
IL	Interleukin (1 - 13)
IDMEM	Iscove's modification of Dulbecco's modified Eagles medium
LPS	Lipopolysaccharide
Mø	Macrophage
mAb	Monoclonal antibody
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony stimulating factor
MIP	Macrophage inflammatory protein
MPO	Myeloperoxidase
NO·	Nitric oxide
NMS	Normal mouse serum
PBS	Phosphate buffered saline
PE	Phycoerythrin
PMN	Polymorphonuclear leukocyte
ROI	Reactive oxygen intermediates
SCF	Stem cell factor
S.D.	Standard deviation of mean
S.E.	Standard error of mean
TG	Thioglycollate
TGF-β	Transforming Growth Factor-β
TNF	Tumour necrosis factor

Chapter 1

INTRODUCTION.

INTRODUCTION.

1. 1 THE MACROPHAGE.

It was Elie Metchnikoff who coined the term "macrophage" (Mø) to describe the large mononuclear phagocytic cells that he observed in inflamed tissues, thus distinguishing them from the more numerous microphages (polymorphonuclear leukocytes; PMN) (Metchnikoff, 1905). Mø are now very well recognized as a major protective element in the host's defense against both invading micro-organisms and against tumour cells. Their potential for injury to the host itself and the delicate balance that must be struck between the protective and destructive roles of this cell have also now been clearly established (Adams, 1992). Although there is great heterogeneity among Mø throughout the body, they all belong to the mononuclear phagocyte system (van Furth, 1972), a system which includes cells believed to share common functional and developmental characteristics, exhibiting most of the following features:

- Distinctive morphology.
- Capacity for phagocytosis.
- Surface expression of Fc and complement receptors.
- Enzyme specificity e.g. non-specific esterase, peroxidase, lysozyme.
- Specific monoclonal antibody binding.

Mø are complex cells and can fulfill a number of often interlinked roles including:

1) The inflammatory response:
    - Recognize and remove inflammatory stimuli.
    - Kill invading micro-organisms
    - Present antigen and invoke protective immune responses.
    - Secrete substances:
        - Enzymes and inhibitors.
        - Lipid mediators.
        - Complement products.
        - Coagulation factors.
        - Matrix proteins.
        - Cytokines and colony stimulating factors.
        - Free radicals.
2) The resolution of inflammation:
    - Tissue debridement and repair.
    - Scavenging of effete cells and cell debris.
    - Stimulation of fibroblasts and control of matrix deposition.
    - Angiogenesis.
3) Protection against tumours.
4) Regulation of haematopoiesis.
5) Also a number of mononuclear phagocytes have specialist functions:
    - Osteoclasts.
    - Kupffer cells.
    - Microglia.
    - Langerhans and dendritic cells.

1. 2 INFLAMMATION AND THE ROLE OF THE MACROPHAGE.

1. 2. 1 The Inflammatory Response.

Perhaps the most crucial role for the Mø is as a central coordinating cell in the inflammatory response where they play an essential role in host defence. The inflammatory response is summarized at the macroscopic level by the well known phrase "calor, rubor, tumor, dolor" and "functio laesa" or loss of function. When viewed at the microscopic level inflammation is a most complex and highly organized process involving cellular and humoral or vascular components. There is close interaction between cells already present at the inflamed site - the vascular endothelial cells, tissue Mø and later fibroblasts, those entering the site, such as platelets, PMN and monocytes as well as more systemically with stimulation of both humoral and cellular immunity. The unique co-ordination of the many cells involved in the inflammatory response requires excellent communication between cells. This is directed by secretion and activation of a highly complicated pattern of mediators including the coagulation system, the complement system, the kinins, the fibrinolytic system and the cytokine network (Dale, 1989a).

The first blood leukocytes to arrive at the site of inflammation are the PMN, seen within two hours (Issekutz, 1981; Haslett, 1989; Doerschuk, 1994). In response to a variety of chemotactic and pro-inflammatory signals they adhere to the vascular endothelium then actively migrate through to the inflamed site where they engulf, kill and digest micro-organisms (Dale, 1989b). Circulating monocytes also accumulate at the inflamed site, following similar pathways to the PMN and once there they mature into inflammatory Mø (van Furth, 1992). The accumulation of monocytes is temporally delayed when compared with PMN but

unlike PMN, monocyte influx does not cease within the first 24 hours, thus Mø become the predominant cell type as inflammation progresses (Issekutz, 1981 and 1993; Adams, 1992; Bellingan, 1996a). Mø were initially thought to function mainly to remove and degrade damaged tissue but their role is far broader, their influence extending throughout the inflammatory response.

In the acute phase of inflammation Mø engulf and kill micro-organisms and through presentation of antigen to lymphocytes, engender highly specific adaptive immune responses to defend the host from invading pathogens (Davies, 1989). During the later stages of inflammation with tissue repair and remodelling, Mø function to debride tissue and in their absence wound healing is impaired (Riches, 1988). Mø secrete growth factors that stimulate fibroblasts, smooth muscle cells and endothelial cells and initiate the fibrotic response characteristic of the repair phase (Leibovich, 1975; Riches, 1988; Davidson, 1992). Not only are Mø a source of growth factors for fibroblasts and other mesenchymal cells but they promote neovascularisation especially in a hypoxic micro-environment (Riches, 1988). Mø also act to remove other inflammatory cells, efficiently phagocytosing effete PMN thus helping damp down the inflammatory response (Haslett, 1994).

1. 2. 2 Mechanisms by Which the Macrophage can Damage the Host.

The inflammatory response can injure the host when initiated or perpetuated inappropriately, either in response to agents that are not threatening to the host or when deployed to excess (Dale, 1989a; Adams, 1992). The Mø can damage tissue by histotoxic enzyme release (Dannenberg, 1975; Adams, 1992), release of free radicals which damage

normal cells (Adams, 1992; McCord, 1993; Halliwell, 1995) and nitric oxide (NO·) which can contribute to the vascular paresis characteristic of septic shock (Szabo, 1995). In addition presentation of antigen may result in inappropriate lymphocyte activation, as well as persistent cytokine secretion (Nathan, 1987; Klareskog, 1988; Khalil, 1989; Harris, 1995). The resolution of acute inflammation is characterized by the clearance of recruited PMN and Mø and the return to normal tissue architecture but often acute inflammation does not resolve, rather it persists, with the development of chronic inflammation and fibrosis, exemplified by a number of diseases including chronic bronchitis and emphysema (Khalil, 1989), glomerulonephritis (Baker, 1994) and rheumatoid arthritis (Koch, 1991 and 1994). The mechanisms underlying the development of chronic inflammation are poorly understood but a hallmark of this is Mø accumulation. Mø are also implicated in other chronic pathological events including atherogenesis, carcinogenesis and granulomatous inflammation which will not be discussed further here. It is clear from this that a knowledge of Mø functions and of the controls on their influx, persistence and removal from the inflammatory site are central to our understanding of the pathogenesis of acute and chronic inflammation.

1. 2. 3 Macrophage Functions in Acute Inflammation.

1. 2. 3. 1 Enzyme Secretion.

Mø secrete a number of enzymes important in the inflammatory response including the antimicrobial enzymes lysozyme (a good marker of Mø activation) (Keshav, 1991) and the cathepsins (Adams, 1992). There is also abundant evidence that activated Mø damage, degrade and remove connective tissue at the inflamed site. Important enzymes in this process are the neutral proteases, elastase, collagenase and

plasminogen activator, although the acid hydrolases will contribute in acidic micro-environments (Riches, 1988; Leenen, 1993). Unless this enzyme secretion is tightly controlled it will damage host tissues and impair organ function. Controls include α_2-macroglobulin which, on exposure to proteolytic enzymes, has a negative feed back effect, inhibiting further protease secretion (Feldman, 1985) and the anti-proteases including α1–anti-protease and plasminogen activator inhibitor (Adams, 1992).

1. 2. 3. 2 Reactive Oxygen Intermediates.

1. 2. 3. 2a Generation of Reactive Oxygen Intermediates.

One of the major antimicrobial mechanisms the Mø possess is the production and release of free radicals (Babior, 1975; Reiss, 1978; Adams, 1992). Free radicals are molecules containing one or more unpaired electrons, the presence of which greatly increase the reactivity of the species. Substances providing electrons to oxygen (or any other electron acceptor) are oxidized whilst the substance gaining electrons is reduced. Molecular oxygen has a strong affinity for four more electrons, when it gains these it also acquires four protons from the solvent and the bond between the two oxygen molecules breaks providing two molecules of water

$$O_2 + 4e^- + 4H^+ = 2H_2O$$

There is an initial kinetic barrier to this complete reduction of oxygen that enzymes with a binding site for oxygen can overcome hence gaining the huge amounts of energy available from providing electrons to molecular oxygen. Oxygen can however sometimes gain unpaired electrons in non-enzymatic auto-oxidations (McCord, 1993; Halliwell, 1995). The gaining of a single unpaired electron produces the superoxide

free radical $O_2^{\cdot -}$; activated phagocytes produce superoxide anions by an enzymatic process in which electrons are transferred from NADPH to molecular oxygen (Weening, 1975; Forman, 1986; McCord, 1993). Transfer of two electrons yields hydrogen peroxide H_2O_2 which itself is not a free radical but is still a powerful oxidant, gaining one further electron yielding water and the hydroxyl radical OH· (Repine, 1979). In the phagocyte, the enzyme superoxide dismutase catalyses the conversion of superoxide radical to hydrogen peroxide and the hydroxyl radical is produced from hydrogen peroxide by the Fenton reaction whereby iron in its ferrous state is oxidized by hydrogen peroxide to yield ferric iron and the hydroxyl radical (Forman, 1986; McCord, 1993). Thus, superoxide, hydrogen peroxide and the hydroxyl radical are all reactive oxygen intermediates (ROI); they are not all free radicals. There are other biologically relevant free radicals not simply derived by the incomplete reduction of oxygen, including hypochlorous acid, NO· and the more related reactive species peroxynitrite (Leenen, 1993).

1. 2. 3. 2b Antimicrobial Action of Reactive Oxygen Intermediates.

The ability of the Mø to kill a wide range of pathogens including Leishmania (Murray, 1982), Toxoplasma, Trypanosoma and Candida (Nathan, 1982) is directly related to its ability to secrete hydrogen peroxide (Adams, 1992). Both PMN and Mø utilize ROI for killing microorganisms, although there are differences between these cell types. PMN possess significant levels of myeloperoxidase (MPO) enabling generation of hypochlorous acid; the role of the peroxidase-hydrogen peroxide-halide system in the Mø, which is relatively deficient in MPO, is more questionable. Mø do however express the inducible form of nitric oxide synthase generating NO·. This is another powerful free radical with

diverse functions including as a vasoactive agent and a neurotransmitter but also with antimicrobial actions, its production being again closely linked to the antimicrobial capacity of Mø (Green, 1990; Fortier, 1992).

1. 2. 3. 2c Deleterious Actions of Reactive Oxygen Intermediates.

There is increasing evidence that free radicals can be damaging to cells and tissues and they have been implicated in a number of disease states, including the "major killers" - cancer and cardiovascular disease (Rice-Evans, 1995) and HIV disease (Greenspan, 1994; Sandstrom, 1994). Reaction of free radicals with non radicals can result in a free radical chain reaction, the most important of these being that of lipid peroxidation (McCord, 1993; Halliwell, 1995). Free radicals can also damage DNA and react together to produce toxic compounds such as peroxynitrite - a product of superoxide and NO· (Schraufstatter, 1987; Halliwell, 1995). A chronic imbalance between the production of ROI and the level of antioxidants has been proposed to result in the cumulative effects of oxidative stress, which may cause disease (Rice-Evans, 1995). There is good evidence that acute free radical induced damage results from excessive phagocyte activation in lung disease and inflammatory bowel disease (Cantin, 1987; Maier, 1993). Experimentally the addition of the free radical generating system xanthine/xanthine oxidase results in acute lung injury in rats that can be blocked by superoxide dismutase (Johnson, 1981). Extracellular fluid from lungs of patients with idiopathic pulmonary fibrosis has below normal levels of the antioxidant reduced glutathione (GSH), whilst phagocytes from these patients release greater quantities of ROI than control phagocytes (Cantin, 1987). Aerosolized glutathione is now in trial as a therapy for pulmonary fibrosis (Maier, 1993).

1. 2. 3. 3 Cytokine Secretion.

The main groups of currently described cytokines are shown in **Table 1. 1**.

Table 1. 1

The Major Groups of Cytokines.

Cytokine Groups	Members
Interleukins:	Molecular messengers acting between the leukocytes (IL-1 to IL-16).
Colony stimulating factors:	(M-CSF, GM-CSF, G-CSF Erythropoietin SCF and IL-3).
Tumour necrosis factor:	(TNF-α and β).
Chemokines:	Low molecular weight chemotactic cytokines.
α chemokines:	Chemokines containing two adjacent cysteine residues (C-C) (including IL-8, MIP-2α, MIP-2β and platelet factor 4).
β chemokines:	Chemokines containing two cysteine residues separated by another amino acid (C-X-C) (include MIP-1α, MIP-1β, RANTES, MCP-1 MCP-2 and MCP-3).
Growth factors:	(erythropoietin, SCF, monocyte chemotractant protein, transforming growth factor, platelet derived growth factor etc.).

Cytokines play a central role, co-ordinating both the inflammatory response and tissue repair. They are low molecular weight proteins that

can act both locally and systemically, binding to specific cell surface receptors, initiating or modulating a wide variety of functions. Since Mø exhibit considerable functional plasticity, the cytokine milieu to which Mø or newly recruited monocytes are exposed will be important in determining the functional and phenotypic states which the cell will acquire. Early in the inflammatory response the dominant forces are the pro-inflammatory cytokines such as TNF-α and IL-1, which have powerful Mø activating potential. During resolution the levels of these cytokines wane and colony stimulating factors and growth factors predominate; Mø phenotype varying accordingly (Leenen, 1993).

1. 2. 3. 3a Pro-inflammatory Cytokines.

TNF-α, and IL-1 are the archetypal pro-inflammatory cytokines, central to the initiation and potentiation of the inflammatory response (Dinarello, 1984, 1986, 1991 and 1993; Tracey, 1986, 1989 and 1994; Cannon 1990; Witsell, 1992). These two cytokines, along with IL-6, are released early in the course of inflammation. After bacterial or lipopolysaccharide (LPS) challenge *in vivo*, TNF-α can be detected within 30 minutes (Tracey, 1986; Natanson, 1989; Collart 1990), IL-1 within 3 hours (Okusawa, 1988) and IL-6 within 6 hours (Gauldie, 1987; Wong, G. 1988). IL-6 differs from TNF-α, and IL-1 in that it does not cause tissue injury, shock and death upon infusion into animals and may act as a protective rather than pro-inflammatory cytokine (Gauldie, 1987; Oritani, 1992; Gillan, 1993). TNF-α, IL-1 and IL-6 are all synthesized and secreted by the Mø, which also express receptors for each of these three key inflammatory cytokines, thus emphasizing the importance of the Mø in inflammation (Heinrich, 1990; Beyaert, 1994). Some of the actions of these cytokines are shown in **Table 1. 2.**

Table 1. 2

Pro-inflammatory Cytokines.

Cytokine	Action	Receptor
TNF-α	*In vitro* Stimulates release of cytokines, ROI and expression of adhesion molecules. Primes and activates inflammatory cells. Promotes phagocytosis. Differentiation factor for monocytes. *In vivo* Endogenous pyrogen. Infusion induces acute lung injury, tissue necrosis, shock and death.	CD 120a (p55) CD 120b (p75)
IL-1	*In vitro* Stimulates release of cytokines, ROI, acute phase proteins and adhesion molecule expression. *In vivo* Endogenous pyrogen. Infusion induces acute lung injury, shock and death.	CDw 121a CDw 121b
IL-6	*In vitro* Stimulates release of acute phase proteins and production of proteinase inhibitor and fibrinogen. Interacts with CSF's to stimulate myeloid proliferation and Mø differentiation. Stimulates B cells. *In vivo* Endogenous pyrogen. Does not cause shock or death upon infusion.	CD 126

TNF-α is a trimeric peptide that binds to two different receptors (Hohmann, 1989). There are two IL-1 proteins, IL-1α and IL-1β, which exist within cells in their pro-forms. The conversion of pro-IL-1β into the active mature form has been shown to be performed by the cysteine protease interleukin-1β converting enzyme (ICE) located on the external cell surface (Cerretti, 1992; Thornberry, 1992; Wilson, 1994; Singer, 1995). Recently ICE deficient mice have been generated that in addition to a major defect in IL-1β synthesis also show an unexpected impairment in IL-1α production (Li, 1995). Only the mature form of IL-1β has activity whereas both forms of IL-1α are active. The predominant monocyte product is IL-1β but as the cell matures into a Mø, IL-1α becomes the major IL-1 protein *in vivo* (Beuscher, 1992). The mechanism of their release is not fully understood, as the IL-1 proteins have no signal peptides thus cannot be released from via the secretory pathway. Hogquist *et al.* have shown that with cell necrosis there is release of both the pro and mature forms of IL-1α but only the inactive pro-IL-1β, whilst apoptotic cell death allows IL-1β to be efficiently processed to its active form and released; the significance of this is not yet clear (Hogquist, 1991a and b).

1. 2. 3. 3b Growth Factors and Colony Stimulating Factors.

In addition to their role in the inflammatory process, cytokines are vital for the growth, repair, differentiation and survival of a range of cell types. This is exemplified by the growth factors, for example - transforming growth factor-β, (TGF-β) (Wahl, 1987; Border, 1990 and 1992; Broekelmann, 1991; Shah, 1992) and the colony stimulating factors M-CSF and GM-CSF (Tushinski, 1982; Warren, 1986; Wiktor-Jedrzejczak, 1990; Hamilton, 1993a and b; Finbloom, 1993; Gillan, 1993). The murine colony

stimulating factor (CSF-1) has direct survival, proliferative and differentiating effects on murine Mø. It has been more difficult to show such an effect with the human counterpart M-CSF on Mø progenitor cell proliferation and monocyte survival. In the murine system CSF-1 will induce more than 90% of bone marrow derived Mø to proliferate whereas less than 5% of human cells will be similarly induced by M-CSF (Cheung, 1992). M-CSF alone is also unable to induce human monocyte to Mø differentiation but anti-M-CSF antibodies inhibit the differentiation-promoting effects of serum, suggesting it does have a part-role (Becker, 1987; Brugger, 1991). The Mø is a significant source of TGF-β at the inflammatory focus and as TGF-β has the ability to induce its own production and it is chemotractant for monocytes, a potential positive feedback loop exists (Border, 1992). **Table 1. 3** shows some of the actions of growth factors and colony stimulating factors.

Table 1. 3

Growth Factors and Colony Stimulating Factors.

Cytokine	Action	Receptor
TGF-β	*In vitro* Regulates fibroblast and epithelial cell growth and extracellular matrix deposition, composition and degradation. Monocyte chemoattractant. Suppresses Mø secretion of pro-inflammatory cytokines.	TGF-β-R
	In vivo Facilitates normal embryonal growth and regeneration following injury. Implicated in many chronic scarring conditions.	

Table 1. 3 (cont.)

Growth Factors and Colony Stimulating Factors.

Cytokine	Action	Receptor
M-CSF (Human) CSF-1 (Murine)	*In vitro* CSF-1 promotes survival, proliferation differentiation and activation of murine Mø. Less clear proliferation and differentiation effect of M-CSF on human Mø progenitors. Promotes pro-inflammatory cytokine release from adherent cultured Mø. *In vivo* Important in osteoclast maturation.	*c-fms* product
GM-CSF	*In vitro* Promotes growth and differentiation of haemopoietic cells. Primes Mø, up-regulates HLA-DR and IFN-γ receptor expression and IL-1ra and plasminogen activator production. Activates PMN. *In vivo* Differentiation and survival of PMN. Important in Langerhans cell differentiation.	CDw 116

Three structurally and functionally related isoforms of TGF-β are expressed by man, of which TGF-β_1, a dimeric protein, is mainly of haematopoietic cell origin (McCartney-Francis, 1994). The colony stimulating factors are a group of glycoprotein hormones that act on the bone marrow stem cells to coordinate the growth and differentiation of the various haemopoietic cells (Weisbart, 1989). Most are monomeric

proteins with the exception of M-CSF and IL-5 which exist as homodimers (Morris, 1991). Many cytokines are involved in the growth and differentiation of other cells of the monocyte lineage. Gene knockout experiments demonstrate that M-CSF and *c-fos* regulated gene products are critically important in osteoclast maturation (Wiktor-Jedrzejczak, 1990) whilst GM-CSF and IL-4 are important in Langerhans cell differentiation. TNF-α and GM-CSF have also been shown to be involved in the differentiation of stem cells into dendritic cells and Mø (Santiago-Schwarz, 1992; Gordon, 1995).

1. 2. 3. 3c IL-4 and Interferon-γ.

Some of the actions of these two cytokines are shown in **Table 1. 4**. In mice T helper cells can be differentiated into the two subsets TH1 and TH2, but most human T cell clones do not fit into these categories (Mosmann, 1986; Modlin, 1993). IFN-γ, produced by TH1 cells, is an important cytokine for Mø function and cellular immune responses (Black, 1987; Kemmerich, 1987; Adams, 1989; Nicod, 1993; Munn, 1995). IL-4, which has powerful modulatory effects on Mø function, is produced by TH2 cells along with IL-5, IL-6 and IL-10. The Mø is a rate limiting effector cell in wound healing (Leibovitch, 1975) which not only secretes but also responds to a number of growth factors like TGF-β and other cytokines including IL-4 that are critical to the successful resolution of the inflammatory lesion (Border, 1992; Davidson, 1992; Allen, 1993). Mø play many vital roles during repair and resolution events, contributing to appropriate fibroblast function including matrix deposition, angiogenesis and to clearance of effete cells and these functions are influenced by a different orchestra of cytokines from those coordinating the acute events of inflammation.

Table 1. 4

IL-4 and IFN-γ.

Cytokine	Action	Receptor
IFN-γ	*In vitro* Stimulates TNF-α and IL-1 production. Monocyte differentiation factor. Induces MHC class II expression.	CDw 119
	In vivo Important for effective cellular immunity, protection against intra-cellular pathogens and for granuloma formation.	
IL-4	*In vitro* Supports B and T cell proliferation. Inhibits pro-inflammatory and MIP-1α cytokine production by Mø. Enhances antigen presentation and MHC class II expression. Down regulates CD14 expression, NO generation and superoxide release.	CDw 124
	In vivo Promotes uptake of pathogens and suppresses chronic inflammation.	

1. 2. 3. 4 Cytokine Receptors and Receptor Shedding.

Cytokines communicate with cells via specific cell surface receptors, the expression of which is closely controlled. Binding of cytokines to their specific receptors may then initiate cellular responses, although receptor expression does not necessarily indicate that they are functionally active. There are now known to be structural similarities between different receptors allowing them to be grouped into families as shown in **Table 1. 5** (Miyajima, 1992a; Bazan, 1993).

Table 1. 5

Cytokine Receptor Superfamilies.

a) **Immunoglobulin superfamily**, with tyrosine kinase activity: IL-1 receptor CDw 121a, M-CSF receptor, Platelet derived growth factor receptor (β subunit) and IL-6 receptor.

b) **Hematopoietin superfamily**, with conserved Trp-Ser-X-Trp-Ser sequence: erythropoietin receptor, growth hormone receptor, prolactin receptor, IL-2 receptor (β and γ subunits), IL-3 receptor (α and β subunits), IL-5 receptor (α and β subunits), GM-CSF receptor (α and β subunits), IL-6 receptor (α subunit), ciliary neurotrophic factor receptor, leukemia inhibitory factor receptor, gp 130, IL-4 receptor (α subunit), IL-7 receptor, IL-9 receptor and G-CSF receptor.

c) **TNF superfamily**, with several cysteine rich domains: both TNF receptors CD120a and CD120b, low affinity nerve growth factor receptor and Fas amongst its members.

There remain a number of other cytokine receptors, including TGF-β and the high affinity receptors for the chemokines, whose structures are unknown and have not been classified in this way as yet.

Many cytokine receptors are composed of more than one protein, for example the GM-CSF receptor (GM-CSFR) consists of an α and a β component, as do receptors for IL-3 and IL-5. These three members of the hematopoietin family, have their own specific α protein but share a common β subunit. The actual mechanism of signal transduction by these receptors is still unclear as the β subunit has no intrinsic kinase activity (Miyajima, 1992b). Similarly the IL-6 receptor consists of two

polypeptide chains - an 80-kD low affinity binding protein (CD126) that has domains representative of both the hematopoietin and the immunoglobulin families and a 130-kD protein, gp 130, that allows high affinity binding of IL-6 to the receptor and is required for signal transduction (Hibi, 1990). Like the GM-CSF/IL-3/IL-5 receptor system, IL-6 receptor shares the signal transducing element gp 130 with other receptors including ciliary neurotrophic factor, leukemia inhibitory factor and oncostatin M (Kishimoto, 1992; Stahl, 1993).

TNF-α has two cell surface receptors, and although commonly co-expressed epithelial cells express more CD120a whereas myeloid cells express more CD120b. Likewise IL-1 has two receptors, CDw 121a - widely expressed on endothelial cells, hepatocytes, T cells and Mø, and CDw 121b - expressed on Mø and some B cells and has not been shown to transduce signal (Sims, 1988; McMahan, 1991; Kikutani, 1995).

A number of receptors are shed from the Mø surface upon activation, including both TNF receptors, the IL-4 receptor, IL-6 receptor and CSF-1 receptor (Porteu, 1990; Kikutani, 1995; Leeuwenberg, 1995; Mullberg, 1995). Shedding is mediated by proteolytic cleavage and for the TNF receptor - CD120a and IL-6R but not M-CSF receptor this shedding has been shown to be inhibitable by a specific metalloprotease (Mullberg, 1995). Most shed receptors act as circulating antagonists but the IL-6 receptor complexed with IL-6 can act as an agonist by binding to gp130 (Fraser, 1993). Receptor loss can also be a consequence of apoptosis with CD16 loss paralleling the onset of apoptosis in PMN (Dransfield, 1994).

1. 2. 3. 5 CD 14.

The Mø is the archetypal LPS binding cell, this binding inducing secretion of pro-inflammatory mediators, up-regulation of adhesion molecule expression and an increase in oxygen consumption. LPS binds to multiple cellular receptors, the best characterized of which is CD14 (Wright, 1990 and 1992; Ferrero, 1993; Zeigler-Heitbrock, 1993; Cohen, 1995). CD14 is a 55kD glycoprotein located mainly on myeloid cells that is anchored to the cell membrane by a glycosyl-phosphatidylinositol (GPI) linkage (Haziot, 1988; Bazil, 1991). LPS binding by CD14 is dependent upon the presence of the plasma protein lipopolysaccharide binding protein (Wright, 1990 and 1992). The mechanism of signal transduction remains unclear as the CD14 molecule has no intracytoplasmic tail. CD14 is not expressed on early myelomonocytic cells but expression can be induced by differentiating agents such as vitamin D3 (Rigby, 1984), more mature cells constitutively express CD14 (Zeigler-Heitbrock, 1993). Differentiation of monocytes into Mø *in vitro* can result in increased, unchanged or decreased expression of CD14 and CD14 expression *in vivo* may be determined by tissue localization with peritoneal Mø expressing higher levels than alveolar Mø and microglial cells (Franklin, 1986; Zeigler-Heitbrock, 1993). CD14 expression changes with the activation state of the cell, in granulocytes and Kupffer cells CD14 expression is up-regulated with activation and TNF-α, IL-1 and IL-6 all increase Mø expression of CD14 (Zeigler-Heitbrock, 1993). LPS stimulation has been reported to both increase and decrease CD14 expression (Zeigler-Heitbrock, 1993) and IFN-γ and IL-4 have been shown to down-regulate CD14 expression on Mø within minutes (Landmann, 1992). Decrease in CD14 expression is mainly by receptor shedding via a proteolytic

mechanism, soluble CD14 can present LPS to cells and can be detected in Mø culture supernatants and in urine from patients (Bazil, 1991).

1. 2. 3. 6 Receptor Expression on Macrophages.

In keeping with their wide range of functions, mononuclear phagocytes express a vast array of receptors (Miller, 1985; Fraser, 1993; Bellingan, 1995). These are listed in **Table 1. 6**.

Table 1. 6

Receptor Expression on the Macrophage.

- **The immunoglobulin superfamily.**
  (e.g. FcR, IL-1R, M-CSFR, MCH I and II and β_2M).
- **The hematopoietin receptor superfamily.**
  (e.g. IL-2R, IL-4R, IL-6R and GM-CSFR).
- **The integrin superfamily.**
  (e.g. CD11a, CD11b, CD11c, CD18, CD29/49d).
- **The selectin superfamily.**
  (L selectin).
- **Matrix component receptors.**
  (e.g. AGE (advanced glycosylation end product receptor) and CD44).
- **Lectin receptors.**
  (e.g. Mø mannose receptor, sialoadhesin).
- **Scavenger Receptors.**
  (type 1 and type II).
- **Soluble receptors.**
  (e.g. IL-4R, IL-6R, both TNF receptors, M-CSFR, CD14)
- MHC

In particular monocytes and Mø express receptors for a number of cytokines including; TNF, IL-1 (Uhl, 1989), IL-2 (Cox, 1990), IL-4 (Feldman, 1990), IL-6, IL-10, M-CSF, GMCSF and IFN-γ (Fraser, 1993; Munck-Petersen, 1990; Bellingan, 1995). The expression of a number of surface receptors is known to alter during Mø maturation, with expression of CD11a/CD18, CD11b/CD18, CD14, Ia and transferrin receptors all increasing with increasing maturity, whilst in the mouse F4/80 is also a maturation marker (Hume, 1983; Miller, 1985; Gessani, 1993). Monocyte production and Mø maturation is closely controlled, being regulated by a number of cytokines including M-CSF, IFN-γ and vitamin D_3 as discussed earlier. Any changes in expression of cytokine receptors during monocyte to Mø maturation will be very relevant to the participation of these cells in the inflammatory response. Activation induces further changes in cytokine receptor expression, in particular it induces receptor shedding as discussed in section 1. 2. 3. 4. Although some of the surface phenotype changes associated with Mø maturation have been analyzed the changes in cytokine receptor expression during the differentiation of these cells has not been documented (Andreesen, 1990).

1. 2. 3. 7 The Macrophage and the Immune Response.

Mø play a part in regulation of virtually all facets of the immune response. They process and present antigens thus stimulating adaptive immune responses by T cells, stimulate B cell reactivity through secretion of cytokines including IL-1 and IL-6 and also mediate suppression of lymphocyte reactivity by secretion of IL-4 and other cytokines (Dale, 1989a; Davies, 1989). Mø, dendritic cells and B cells are the major antigen presenting cells (Austyn, 1990; Steinman, 1991; van Vugt, 1993a). For appropriate presentation, antigen must be taken up and processed to the

form of an antigenic peptide such that it can be combined with MHC class I or II molecules on the cell surface (Chain, 1993). Binding of antigen specific cells expressing the T cell receptor, CD4 or CD8 molecules and adhesion molecules along with delivery of an appropriate co-stimulatory signal by the antigen presenting cells then elicits a positive T cell response (Chain, 1993). Although Mø are competent antigen presenting cells, only a proportion express class II molecules and their role may be restricted to evoking a memory T cell response, in contrast with dendritic cells which are by far the most effective antigen presenting cells for provoking primary T cell responses (Steinman, 1991). The relative antigen presenting activity of dendritic cells and Mø *in vivo* needs to be clarified (Chain, 1993). Dendritic cells are non-phagocytic and possess few lysosomes suggesting a limited capacity for intracellular protein degradation. Thus despite their great efficacy in antigen presentation it has been proposed that they are relatively inefficient at processing antigens. Suggestions as to how this is overcome *in vivo* include the possibility of close cooperation between Mø and dendritic cells and/or the possibility that peripheral antigen processing cells could mature into dendritic cells (Steinman, 1991; Chain 1993). Certainly dendritic cells and Mø seem to be derived from a common progenitor cell and there is evidence that not only can dendritic cell differentiation be selectively promoted from a common Mø/dendritic stem cell but that dendritic cell morphology may be a transient feature of cells of the monocyte/Mø lineage (Santiago-Schwarz, 1992). In the peritoneum there are normally only 1 - 2% dendritic cells and only 13% of the resident peritoneal Mø are Ia positive, with activation the percentage of Ia expressing Mø increases to 45% (Kimberly, 1992; van Vugt, 1993a). The relative contribution of these Ia positive Mø to the development of adaptive immune responses is not

known. Certainly both dendritic cells and Mø have been shown to traffic from tissues to the draining lymph nodes (Rosen, 1990, van Furth, 1992, Lan, 1993, Xia, 1995; Bellingan 1996a). In the lung, alveolar Mø have been shown to down-regulate the antigen presenting capacities of pulmonary dendritic cells; thus the role of Mø migration may even be as immune modulator rather than immunostimulator (Holt, 1993; Xia, 1995).

1. 3 THE LIFE OF THE MACROPHAGE.

1. 3. 1 Origin.

Aschoff in 1924 grouped the Mø into the reticuloendothelial system, a wide ranging system including monocytes, Mø, histiocytes, fibroblasts, endothelial cells and reticular cells on the basis of their structural association (Aschoff, 1924). The reticuloendothelial classification was replaced in 1969 with the concept of the mononuclear phagocyte system, which includes cells believed to share common functional and developmental characteristics (van Furth, 1972). Mø are found in every tissue in the body and along with this wide distribution there was found to be considerable Mø heterogeneity. Investigation of Mø origins and development sought to determine whether this heterogeneity arose as a result of specific Mø sub-populations or a complex function of site, degree of differentiation and state of activation. Mø are originally derived from the bone marrow. The multipotent haematopoietic stem cell (CFU-S) yields the granulocyte-macrophage colony forming cell which divides under the influence of the appropriate colony stimulating factors to give rise to the monoblast. The monoblast, defined in 1975 by van Furth and colleagues, divides once to yield two promonocytes which further divide to give rise to the monocyte which leaves the bone marrow to enter a circulating and marginated pool (van Furth, 1992). van Furth and

colleagues also demonstrated that circulating monocytes were a source for resident peritoneal Mø, Kupffer cells, splenic Mø, alveolar Mø and inflammatory Mø. Thus, the single lineage model of Mø development suggests that under normal steady state conditions monocytes, derived from bone marrow precursors, migrate to tissues and body cavities and there differentiate into Mø (van Furth, 1992; Ginsel, 1993). However recent evidence suggests that there is also local cell division of Mø depending on the tissue site (Dougherty, 1984; Ginsel, 1993). The extent to which local proliferation occurs is a matter of contention; van Furth and colleagues maintain that newly arrived monocytes have only a limited capacity for division (van Furth, 1992), whilst others present evidence that extensive local proliferation occurs (Volkman, 1976; Bouwens, 1986; Melnicoff, 1988a; Ginsel, 1993).

Monocytes can be shown to mature and differentiate into Mø, undergoing extensive changes in surface receptors, enzyme activity and secretory ability. This functional and phenotypic plasticity is used as evidence that the single lineage model holds true, although it is difficult to explain all the intra-population heterogeneity observed in this way. Depletion of local Mø populations and use of parabiotic animals suggested that the bone marrow was not (entirely) the source for replenishment of tissue Mø populations (Volkman, 1976; Bouwens, 1986; Sawyer, 1986; Tarling, 1987). For these reasons a dual lineage model for Mø development has been proposed which suggests resident Mø populations are maintained mainly by local proliferation and are thus distinct from circulating monocytes and inflammatory Mø (Ginsel, 1993).

1. 3. 2 Heterogeneity.

The mononuclear phagocyte system classification includes resident peritoneal Mø, alveolar Mø, Kupffer cells, splenic Mø, circulating monocytes, exudate or inflammatory Mø and a number of other specialized cells such as microglia and osteoclasts. This demonstrates the great diversity within the classification of Mø-like cells. Part of this heterogeneity arises due to localization at different sites within the body. A clear example of this is seen with the differences in Fcγ and C3 receptor and other surface antigen expression on circulating monocytes, resident peritoneal Mø and alveolar Mø (van Furth, 1992). This heterogeneity extends not only to phenotype but functional abilities as well as antigen and enzyme expression (van Furth, 1992).

Within Mø all located in one compartment there is also considerable variation - intra-population heterogeneity. Some of this may be due to mixing of Mø populations such as resident Mø with monocytes newly called in by an inflammatory stimulus. Another source of heterogeneity is the phenotypic and functional changes associated with differentiation and activation since a population is unlikely to be synchronous with respect to either of these processes.

1. 3. 3 Differentiation.

Some investigators define development of the monocyte from its precursor promonocyte and monoblast as maturation whilst generation of diversity is defined as differentiation (Leenen, 1993). In contrast with activation, both maturation and differentiation are irreversible steps in the life of the Mø and this contrasts with activation. Differentiation includes, cytochemical changes, acquisition of new functional capacities,

along with an increase in size and altered surface phenotype (Dougherty, 1984). The process of differentiation is tightly regulated by a number of cytokines including M-CSF, GM-CSF, IL-3 and TNF-α as well as by other agents including steroid hormones (Dougherty, 1984; Leenen, 1993). Exposure of cells to different environments will further alter their characteristics, for example exposure to high or low oxygen concentrations (Albina, 1995). Within each specific micro-environment, the matrix, the local cytokine milieu and a host of other factors will combine to induce specific Mø differentiation characteristics.

1. 3. 4 Activation.

Further heterogeneity is associated with differing Mø activation states, although there is still controversy about the terms "primed" and "activated" when applied to Mø. The concept of Mø activation was introduced in the 1960's by Mackaness who demonstrated that Mø resistance to intracellular parasites was brought about by the action of T lymphocytes. After exposure to lymphokines derived from sensitized T cells, Mø gained the ability to kill a range of intracellular pathogens and were described as "angry" or "activated" cells (Mackaness, 1970). "Activation" is applied to intrinsic adaptive changes undergone by Mø to enable it to respond, enhancing microbicidal capacity (North, 1978) and cytotoxicity for tumor cells (Adams, 1989). One potential source of confusion is that changes in effector molecule expression during Mø differentiation may allow definition of Mø with the capacity for a particular function (Cohn, 1978; Karnovsky, 1978). However receptor expression does not necessarily equate with functional competence. Thus Adams has suggested that Mø activation should be viewed in terms of competence to perform a complex function, thereby distinguishing

between potential capacities of the cell such as expression of a specific receptors and functions such as the killing of microbes, antigen presentation or chemotaxis (Adams, 1989). There is not one single "activated" state for Mø. A Mø which, through various signals from its environment, has acquired the ability to perform one particular function need not be capable of performing a different complex function (North, 1978; Campbell, 1984; Adams, 1989). In terms of the inflammatory response, considerable Mø functional heterogeneity is likely to exist due to a number of factors including; the cytokine profile to which the cells are exposed, the inciting stimulus, the site of inflammation and stage of the inflammatory response (Dougherty, 1984; Lemaire, 1991; Leenen, 1993). For the steady state resident Mø, the local environment normally provides for a low activation state, whilst the inflammatory Mø at the inflamed site will be some way down the activation pathway(s).

Whatever the origin of Mø heterogeneity, clear distinctions exist between monocytes, resident Mø and inflammatory Mø, which differ from each other in their expression of a range of receptors and enzymes (van Furth, 1992) as well as their ability to phagocytose and kill pathogens (Cohn, 1978; North, 1978; Lepay, 1985; Black, 1987; Kemmerich, 1987).

1. 3. 5 Monocyte Migration - Adhesion and Chemotaxis.

Monocytes migrate into acute inflammatory reactions in response to many different stimuli, listed in **Table 1. 7** (Wahl, 1987; Riches, 1988; Schall, 1990; Issekutz, 1993; Kasama, 1993; Meurer, 1993). Monocytes leave the circulation under the influence of chemotactic signals and accumulate at the site of acute inflammation. This emigration requires transendothelial migration which, as with the PMN, involves multiple

stages including rolling along then firm adhesion to the endothelium, allowing traversing of the junctional space (Meerschaert, 1995). Again as with PMN, L-selectin (CD62L) is involved in the initial rolling (Spertini, 1992) while the β_2 integrin family member CD11/CD18 and β_1 integrin family member CD49d/CD29 or VLA-4 are both involved in tight binding to endothelial cells (Meerschaert, 1995). The platelet-endothelial cell adhesion molecule-1 (PECAM-1, or CD31) is required for movement of monocytes through endothelial intercellular junctions (Muller, 1993; Liao, 1995). The adhesion molecules involved in the migration of Mø from tissues to lymph nodes have not been elucidated.

Table 1. 7

Monocyte Chemotactic Agents.

Plasma-derived Factors	**Cell-derived Factors.**
C5a and C5a des arg	Platelet factor 4
Fibrinopeptides	Leukotriene B_4
IgG proteolytic fragments	**Cytokines.**
Thrombin	IL-1
Extracellular Matrix-derived Factors.	TNF
Collagen and its fragments	IFN-γ
Elastin and its fragments	RANTES
Fibronectin fragments	MIP-1α
Bacterial-derived Factors.	MCP-1
LPS	TGF-β
fMLP	

There is great diversity among chemotactic signals. Many of these agents, for example collagen type 1 and fibronectin proteolytic fragments, show specific chemotactic activity for Mø but not for PMN. Similarly both C5a des arg and C5a are chemotactic for monocytes, whilst the conversion of C5a into C5a des arg reduces the PMN response 15 fold. This contrasts with IL-8 which is an extremely potent chemotactic agent for the PMN with little effect on monocytes.

1. 3. 6 Cell Culture Systems for Macrophage Study.

Peripheral blood monocytes can be isolated by a variety of techniques including density gradient centrifugation, adherence, or countercurrent centrifugal elutriation (CCE). Monocytes cultured *in vitro* in the presence of serum, either adherent to tissue culture wells, or in suspension in hydrophobic Teflon foils (Johnson, 1977; Andreesen, 1983; Becker, 1987; Helinski, 1988) undergo a number of functional, biochemical and morphologic changes consistent with differentiation or maturation into Mø (Becker, 1987). Cultured monocytes have been shown to become functionally competent phagocytes (Helinski, 1988). Monocytes in culture show increased intracellular levels and secretion of lysozyme, 5' nucleotidase, tissue transglutaminase, creatine kinase, cAMP-dependent protein kinase 1 and non-specific esterase activity, but lose MPO, paralleling the process of Mø differentiation *in vivo* (Johnson, 1977; Andreesen, 1983; Riches, 1988). Along with these functional alterations, monocytes become bigger, more granular and have altered cell surface antigen expression during *in vitro* differentiation (Becker, 1987; Helinski, 1988; Gordon, 1995). Monocyte derived Mø in *in vitro* culture gain new expression of CD16 and CD51 and increased expression of CD11a, CD11b, CD11c, CD14, CD71 and HLA-DR, as do *in vivo* Mø (Andreesen, 1990;

Gessani, 1993; Bellingan, 1993; Zang, 1994). These changes indicate that human peripheral blood monocytes mature into functional Mø *in vitro* whether they are cultured adherently or in suspension (Andreesen, 1983; Becker, 1987) and this mimics the functional, biological and morphological changes accompanying *in vivo* maturation of monocytes passing from the blood stream into tissue in response to inflammatory stimuli (Riches, 1988).

In the absence of serum Mø differentiation does not occur, monocytes fail to increase in size, or gain new surface molecule expression (Andreesen, 1990). There has been considerable interest in what serum factor(s) may cause this differentiation. Musson demonstrated that a factor for survival/differentiation co-isolated with albumin and several workers have shown that for murine Mø, CSF-1 is an important differentiation co-factor but that a number of other cytokines, including GM-CSF, IL-1, IL-3, IL-4, IL-6, TNF-α and IFN-γ were all ineffective (Tushinski, 1982; Becker, 1987; Brugger, 1991). The role of M-CSF in human cells is less clear, Munn *et al.* finding it was an essential survival factor for monocytes whilst Cheung *et al.* demonstrated little effect and Brugger *et al.* showed that it was unable to directly induce differentiation (Brugger, 1991; Cheung, 1992; Munn 1995). Other factors implicated include vitamin D_3 and there is evidence that adhesion via fibronectin receptors to insolubilized fibronectin also induces monocyte differentiation (Riches, 1988). With this uncertainty, a defined "maturation medium" is not in existence and serum, with the inherent variability entailed, is used as standard.

1. 3. 7 *In Vivo* Monocyte/Macrophage Kinetics.

1. 3. 7. 1 Non-inflamed Steady State.

Most studies on monocyte/Mø kinetics have been performed on the mouse. In the non-inflamed steady state monocytes remain in the bone marrow for less than 24 hours before entering the circulation, where they have a half life of approximately 17 hours (van Furth, 1973). The pool of circulating and marginating monocytes in an adult mouse is estimated at 0.62 and 0.92 x 10^6 cells respectively (van Furth, 1992). This compares with 9 x 10^6 tissue Mø in the liver and 2.4 x 10^6 in the peritoneal cavity. As these pool sizes remain constant there must be a constant disappearance of monocytes and/or Mø (Richardson, 1994). Should tissue Mø be replenished from circulating monocytes (single lineage model) then approximately 1.5 x 10^6 Mø must be removed per day (van Furth, 1992). Alternatively, if tissue Mø are mainly replaced by self replication, 1.5 x 10^6 circulating monocytes must then be removed per day, with a much lower rate of Mø removal, since typically less than 5% of tissue Mø are seen to be synthesizing DNA. Certainly the half life of the monocyte is considerably shorter than that of resident tissue Mø. Rosser reported that after adoptive transfer of radiolabelled resident peritoneal Mø more than half the labelled cells could be recovered in the peritoneal lavageate 2 weeks after cell transfer (Rosser, 1970). van Furth concluded that the mean turnover times for different Mø populations varied between 3.8 days for Kupffer cells and 14.9 days for peritoneal Mø (van Furth, 1992).

1. 3. 7. 2 Giant Cell Formation.

One of the hallmarks of the granulomatous response to infection or inflammation is the formation of multinucleate giant cells. Giant cells occur in association with fungal or mycobacterial infection or with certain

forms of chronic inflammation as characterized by sarcoidosis or the response to a foreign body. It is understood that multinucleate giant cells are formed by Mø fusion but the cause and mechanism of this is unclear (Sone, 1981; Enelow, 1992). In culture, multinucleate giant cells are not observed until at least the 4th day, consistent with the requirement for these cells to mature into Mø (Chow, 1989). In suspension cultures no cell fusion could be detected at all (Enelow, 1992). The cells of certain human donors have a marked tendency to giant cell formation, the reason for this is unknown (Hassan, 1989; Enelow, 1992). Agents known to induce multinucleate giant cell formation include, vitamin D_3, concanavalin-A, IL-3, GM-CSF, IFN-γ and phorbol myristate acetate (PMA) (McInnes, 1988; Hassan, 1989; Enelow, 1992; Takashima, 1993). Both a positive (McInnes, 1988) and negative (Enelow, 1992; Takashima, 1993) effect of IL-4 on giant cell formation has been reported. The usual rate of giant cell formation in the absence of a specific stimulus is low and this does not represent a major fate of Mø.

1. 3. 7. 3 Acute Inflammation.

During inflammation the production of monocytes increases due to both shortening of the promonocyte cell cycle times and to an increase in the absolute numbers of promonocytes. This results in a doubling of the number of monocytes entering and leaving the circulation during the first few days of acute inflammation (van Furth, 1992). The bulk of Mø accumulation at the inflamed site is due to an influx of monocytes (Issekutz, 1981 and 1993; van Furth, 1992). A number of investigators have tackled the question of the kinetics of monocyte influx into inflammatory foci and the mechanisms underlying this (Issekutz, 1981 and 1993; Rosen, 1990; Spertini, 1992; van Furth, 1992; Meerschaert, 1995).

Monocyte accumulation at the acutely inflamed site often begins early, certain stimuli can elicit substantial accumulation within 1 hour of the onset of inflammation (Issekutz, 1981). The rate of monocyte accumulation can peak at any time between 4 and 18 hours after the insult (Doherty, 1988) but immigration continues for at least 24 hours depending upon the stimulus (Paz, 1962; Perper, 1974; Issekutz, 1981 and 1993). This contrasts with the PMN which migrates into the acute inflammatory lesion in vast numbers over the first 6 hours but thereafter influx virtually ceases (Haslett, 1989). This, coupled with the relatively short life span of the PMN means that the monocyte becomes the predominant cell after 12 to 48 hours depending on the stimulus (Issekutz, 1981; Doherty, 1988). Labelled monocytes have been used to identify the migration kinetics into inflammatory foci in the lung (Doherty, 1988), peritoneum (Rosen, 1990), skin and footpad (Issekutz, 1981; van Furth, 1992). Resident peritoneal Mø labelled and returned intravenously have been shown to be a useful surrogate for circulating monocytes as, unlike exudate derived or formalin fixed Mø, these cells can migrate into acute inflammatory lesions (Rosen, 1990).

1. 3. 7. 4 Resolution of Inflammation.

The method of clearance of inflammatory Mø during the resolution of acute inflammation is not clear. Mø from inflammatory lesions can migrate to the draining lymph nodes but the importance of this as a mechanism of clearance is unknown (Lan, 1993). Both the mesangial cell, a Mø-like professional phagocyte in the kidney, and the PMN, die by apoptosis and are cleared by local phagocytes (Baker, 1994). Thus, the Mø could meet its fate mainly by death *in situ* and be cleared by the remaining tissue Mø. Although adoptive transfer of labelled Mø has also

been used to track the migration of Mø into inflamed sites, such a study of the kinetics of inflammatory Mø during the resolution of inflammation has not been performed.

The factors that cause Mø to be cleared from the inflamed site and the timing of this are not well understood, however, since a failure to clear Mø from the inflammatory site could lead to the development of chronic inflammation, knowledge of these factors could be of central importance to our understanding of the inflammatory response.

1. 3. 8 Fate of the Macrophage *In Vivo.*

Little is known about the ultimate fate of monocytes and Mø. Mangan *et al.* have shown that, in the absence of pro-inflammatory stimuli, monocytes *in vitro* die by apoptosis (Mangan, 1991a and b). Recently Munn *et al.* suggested that early exposure to IFN-γ may be required for monocytes and Mø to undergo apoptosis (Munn, 1995). Mø have been shown to die by apoptosis in response to a number of signals, including NO·, pathogens, silica and toxins (Waring, 1990; Zychlinsky, 1992; Sarih, 1993a and b), but the physiological significance of this is unknown. As with mesangial cells and PMN (Baker, 1994), it is possible that inflammatory Mø die at the inflamed site. Migration of Mø to local lymph nodes has been demonstrated and as Mø are uncommon in efferent lymph it is possible that they die there (Rosen, 1990; van Furth, 1992; Lan, 1993; Thepen, 1993). The relevance of Mø migration to local nodes is unclear and the importance of this route of clearance over death in the tissues is unknown. There is evidence that lung Mø leave via the airspaces but this is unlikely to be a major route for elimination (Nicol, 1958). The possibility of different clearance mechanisms for resident

tissue Mø and inflammatory Mø during the resolution of inflammation has not been addressed.

1. 4 CELL DEATH.

All cells die. Two main forms of cell death have been described, necrosis and apoptosis. As certain cells have been shown to have a genetic programme controlling the onset of apoptosis this form of cell death is also known as programmed cell death. The causes, controls, characteristics and consequences of apoptosis and necrosis are markedly different and are reviewed below.

1. 4. 1 Necrosis.

Necrosis occurs where there are gross perturbations in the cellular environment such as trauma, large fluctuations in temperature or pH, or in the presence of lethal concentrations of metabolic poisons. A common pathological event leading to necrosis is vascular occlusion leading to lack of perfusion of cells, the resultant hypoxia causes cellular necrosis within minutes, this is evident by light microscopy within hours (Jennings, 1975 and 1978). Initially reversible changes including mild cellular and mitochondrial swelling, lead to irreversible events including gross swelling, rupture of endoplasmic reticulum, disruption of internal organelles and finally plasma membrane disruption (Trump, 1976). Accompanying necrosis there is usually evidence of a significant acute inflammatory reaction (Wyllie, 1988). These morphological changes are accompanied by a fall in ATP levels with a loss of function of vital ion pumps leading to loss of potassium to the extracellular environment whilst sodium and water enter the cell (Trump, 1975). A number of enzymes are activated leading to hydrolysis of membrane phospholipids

and proteins, potentiating the cellular disruption (Farber, 1973). Necrosis does not occur in a developmental context and unlike apoptosis, does not require expression of new mRNA (Cohen, 1993; Schwartz, 1993).

1. 4. 2 Apoptosis.

1. 4. 2. 1 Morphology.

The first descriptions of cell death as a positive contribution to normal vertebrate development were made relatively recently (Glucksmann, 1951; Saunders, 1966). The term apoptosis was first introduced in 1972 by Kerr describing a form of cell death morphologically distinct from necrosis (Kerr, 1972). It is now believed that programmed cell death is a normal default pathway for most living cells, protecting the organism from uncontrolled cell proliferation (Raff, 1992). Apoptosis is characterized by both nuclear and cytoplasmic condensation (Kerr, 1972), which is strikingly rapid and seen clearly by time-lapse video microscopy (Russell, 1972). Initially nuclear condensation can be seen underlying the nuclear membrane, then progressing to involve the entire nucleus (Cohen, 1992). Later the nucleus can fragment into numerous smaller spheres. Within the cytoplasm the endoplasmic reticulum is seen to dilate despite the cell's great decrease in volume. Some of the more superficial dilated ducts fuse with the plasma membrane giving it a pitted appearance on scanning electron microscopy (Wyllie, 1988). The plasma membrane retains its integrity well into the apoptotic process although there is loss of specialized surface structures, loss of cell-cell contact and blebbing of membrane bound bodies (Cohen, 1992). Unlike necrosis there is no associated acute inflammatory reaction even in the face of significant levels of apoptosis (Gerschenson, 1992). In the absence of

phagocytic cells to engulf it, the apoptotic cell undergoes further disintegration, a stage called secondary necrosis.

1. 4. 2. 2 Biochemical Changes Associated with Apoptosis.

Accompanying these morphological changes there are distinct enzymatic processes, the most characteristic being activation of an endonuclease which acts to cleave DNA into approximately 180 base pair oligonucleosomes (Wyllie, 1980; Arends, 1990; Pietsch, 1993). Although apoptosis has been reported to occur in the absence of DNA laddering (Gromkowski, 1986 and 1988), electrophoretic analysis of DNA - showing a characteristic ladder pattern - remains a powerful method of distinguishing apoptotic and necrotic death (Wyllie, 1980; Gerschenson, 1992). In addition to the activation of nuclear enzymes, apoptosis is also associated with activation of other enzymes, including tissue transglutaminase (Fesus, 1987) and ICE (Hogquist, 1991a; Miura, 1993; Wang, 1994). Other changes on the surface of apoptotic cells include phosphatidyl serine translocation from the inner to outer membrane (Fadok, 1992a; Martin, 1995) and shedding of CD16 (Dransfield, 1994).

1. 4. 2. 3 Apoptosis: Cytokines and Serum Survival Factors.

Not only do cytokines influence cellular growth, development and function but they are also important in controlling cell death. There is much evidence that certain cytokines can promote or inhibit apoptosis itself, some examples are given in **Table 1. 8** (GM-CSF - Brach, 1992: TNF-α - Colotta, 1992; Grell, 1994; Polunovsky, 1994: IL-2 - Duke, 1986: IL-3 - Rodriguez-Tarduchy, 1990: IL-4 - Rodriguez-Tarduchy, 1991: IL-5 - Stern, 1992: IL-6 - Afford, 1992). Indeed Raff postulates that without continual cell - cell communication all cells will undergo apoptosis (Raff, 1992).

The concept of cell death in the absence of survival factors is thus well established.

Table 1. 8

Cytokines can Promote or Inhibit Apoptosis.

Promotion of Apoptosis		**Inhibition of Apoptosis**	
Cytokine	**Cell**	**Cytokine**	**Cell**
TNF-α	PMN (early)	GM-CSF	PMN
	Endothelial cells	IL-2	T cells
	Tumour cells	IL-3	Haemopoietic cells
IL-6	PMN	IL-4	Haemopoietic cells
	U937 cells	IL-5	Eosinophils

Monocyte and Mø survival had already been shown to be dependent on CSF-1 in murine cells in the absence of serum (Tushinski, 1982; Becker, 1987; Brugger, 1991). For human monocytes, serum is of critical importance to *in vitro* survival, an effect which is partially abrogated by adherence (Johnson, 1977). In suspension cultures in the absence of serum, survival rates of 1 - 12% have been reported over 5 days whilst corresponding recovery rates in the presence of serum are 30 - 75% (Andreesen, 1983 and 1990; Helinski, 1988; Brugger, 1991; Mangan, 1991a). Improved monocyte recovery rates can be achieved by adherent culture and the addition of serum doubles cell recovery at 5 days (Becker, 1987). Mangan *et al.* have shown that in the absence of serum monocytes die by apoptosis and that this is delayed by the addition of pro-inflammatory cytokines, although interestingly not by monocyte chemotactic cytokines (Mangan, 1991a and b). They did not examine the effect of cytokines on

Mø survival. The effect of serum on Mø survival seems to be less pronounced and the induction of apoptosis by serum withdrawal has not been reported.

1. 4. 2. 4 Role of Free Radicals in the Induction of Apoptosis.

There is now growing evidence that free radicals and ROI can induce not only necrotic, but also apoptotic cell death; low levels inducing apoptosis whilst high levels induce necrosis (Lennon, 1991; Fernandes, 1994). Apoptosis can be induced in a wide variety of cells by different types of oxidative stress including ionizing radiation (hydroxyl radical production) TNF-α (cellular ROI stimulation) and exogenously added ROI (hydrogen peroxide) (Abello, 1994; Forrest, 1994; Sarafian, 1994; Hannah, 1995). In addition, the induction of apoptosis in different cells can be blocked by antioxidants, even when the apoptotic stimulus was apparently ROI independent for example, apoptosis resulting from growth factor withdrawal (Sandstrom, 1993; Mayer, 1994; Sarafian, 1994; Troy, 1994), suggesting that ROI may be mediators of the apoptotic process. Despite the fact that phagocytes produce free radicals, an excess of ROI is detrimental to these cells and their armoury of antioxidants has been shown to be of critical importance to preserving their function (Baehner, 1977). Roos and co-workers showed that normal PMN and monocyte function during oxidative stress (phagocytosis-induced ROI production and exogenous hydrogen peroxide generation) depended on intact antioxidant protective systems (Roos, 1979 and 1980; Voetman, 1980). Phagocytes have developed several systems to protect themselves against oxidative stress. Superoxide dismutases catalyze the conversion of superoxide into hydrogen peroxide which is then degraded to water and oxygen by the action of the enzyme catalase. GSH produced as a part

of the glutathione redox system protects cells by reacting with free radicals and peroxides (Roos, 1979; Fernandes, 1994). Other antioxidants that protect cells from apoptotic cell death include the vitamin E group of compounds, the major one being α tocopherol and it's synthetic soluble analogue Trolox (Sandstrom, 1993; Forrest, 1994; Mayer, 1994; Troy, 1994; Hannah, 1995). They act to block free radical chain reactions by scavenging the intermediate peroxyl radicals, the tocopherol radical being much less reactive than the corresponding lipid peroxyl radical. Vitamin C is also important in the antioxidant system in that it recycles the tocopherol radical (Halliwell, 1995).

1. 4. 2. 5 Intra-cellular Signalling in Apoptosis.

The signal pathways leading to apoptosis are incompletely understood but as multiple signals can induce apoptosis in a cell, it is likely that these signals will mediate their effects though distinct signal transduction pathways (Schwartz, 1993). A rise in intracellular free calcium seems to be a critical event for apoptosis in many cells but this is neither specific for cell death, being involved in signalling for a plethora of cellular activities, nor is it an absolute requisite for the induction of apoptosis (McConkey, 1989; Rodriguez-Tarduchy, 1990). Indeed in PMN, calcium ionophores induced a dramatic slowing of apoptosis (Whyte, 1993). Other intracellular signalling moieties have also been implicated including the inositol phosphate, IP3 and cAMP (Berridge, 1989; McConkey, 1990). cAMP promotes the activity of protein kinase A (Soderling, 1990), activation of which promotes apoptosis. Protein kinase C activation - with phorbol esters - prevents apoptosis, whilst inhibition of protein kinase C with H7 increases apoptosis (McConkey, 1990). It needs to be emphasized that the exact mechanism(s) whereby such a variety of

environmental and genetic factors act to trigger cell death is not clearly understood. Whether there is one "final common pathway" is unknown. There is even dispute as to whether the morphologic changes in the nucleus are central to the apoptotic process or simply represent an epiphenomenon as cytoplast death has many features of apoptosis (Jacobson, 1994; Henkart, 1995).

1. 4. 2. 6 Genetic Control of Apoptosis.

There is much evidence that for the normal functioning of an organism, cell death is genetically controlled, hence the term "programmed cell death" (Lockshin, 1965). Studies of the nematode *Caenorhabditis elegans (C. elegans)* have shown that there is genetic control of cell death, engulfment of these dead cells and the degradation of the cellular debris (Ellis, 1991). The genes *ced* (cell death abnormal) 3 and *ced* 4 acting to cause programmed cell death, *ced* 1, 2, 5, 6, 7, 8 and 10 all control engulfment while *nuc*-1 directs the degradation of nuclear material in the dead cells (Hedgecock, 1983; Ellis, 1986; Yuan, 1990). Unique is *ced* 9 which acts to prevent cell death (Hengartner, 1992 and 1994). This programmed cell death has many of the morphological characteristics of apoptosis although there is still dispute as to whether they are truly one and the same (Schwartz, 1993; Henkart, 1995).

Although our understanding of the control of mammalian cell death, especially its genetic control, is not as well advanced as it is in nematodes, there are indicators that similar genetic controls exist. In mammalian cells ICE, the mammalian homologue of *ced*-3 (Miura, 1993), is associated with apoptosis and *bcl*-2, the human homologue of *ced* 9 has been shown to protect mammalian cells from apoptosis (Vaux, 1992;

Hengartner, 1994). Other mammalian genes including *p53, c-fos, c-myc* and *abl* have also all been implicated in the apoptotic process (Buttyan, 1988; Yonish-Rouach, 1991; Martin, 1995). For many cells there is an absolute requirement for protein synthesis in the apoptotic process; RNA and protein synthesis inhibitors completely blocking apoptosis (Cohen, 1984; Wyllie, 1984; Schwartz, 1993), providing evidence for a genetic switch for apoptosis. It is noteworthy that some cells (PMN and Mø) undergo apoptosis when exposed to RNA or protein synthesis inhibitors, suggesting that these cells have continuous synthesis of an anti-apoptotic protein, inhibition of which leads constitutively to apoptosis, the machinery for this being in place in the absence of protein synthesis (Waring, 1990; Haslett, 1994; Polunovsky, 1994).

1. 4. 2. 7 *bcl*-2 and the *bcl*-2 Family in Apoptosis.

The oncogene *bcl*-2 is of interest because of its proposed anti-apoptotic and anti-oxidant roles. Transfer of *bcl*-2 expression into a cell prolongs survival by preventing apoptosis in a variety of systems including hydrogen peroxide, chemotherapeutic, heat shock and ionizing radiation induced apoptotic cell death (Reed, 1994). The exact mechanism by which this is achieved is unclear but expression of *bcl*-2 prevents oxidative stress induced apoptosis across cell types and across phyla (Hockenbery, 1993; Buttke, 1994; Sarafian, 1994). Although initially suggested to act at the level of the mitochondrial membrane (Hockenbery, 1990), the distribution of the *bcl*-2 gene product has been shown to be much wider than this; *bcl*-2 expression can also prevent apoptosis in cells lacking mitochondrial DNA (Jacobson, 1993), thus *bcl*-2 does not seem play a role in oxidative phosphorylation. It also blocks the cellular accumulation of lipid peroxides and restores growth in cells deficient in manganese

superoxide dismutase (Hockenbery, 1993). Since different antioxidants can be substituted for *bcl*-2 expression and still prevent apoptosis induced by growth factor depletion (Buttke, 1994), it has been proposed that the protective action of *bcl*-2 is by inhibiting generation or action of ROI. Recent work with cells in very hypoxic conditions shows that although ROI-induced apoptosis is thus inhibited, a protective effect of *bcl*-2 on non-ROI-induced apoptosis persists (Jacobson, 1995; Shimizu, 1995). Thus although this does not exclude any effect of *bcl*-2 on ROI induced death, it does indicate that ROI are not required for cell death and that *bcl*-2 protection is broader that just protection against ROI mediated mechanisms alone.

A number of gene products share significant sequence homology with Bcl-2, including Bax, Bcl-x_S, Bcl-x_L and Bak (Reed, 1994; Chittenden, 1995; Kiefer, 1995) and have been found to have a role in apoptosis. Bax and Bcl-x_S primarily act to enhance apoptotic death whilst Bcl-x_L acts like Bcl-2 to delay apoptosis (Chittenden, 1995; Kiefer, 1995).

1. 4. 2. 8 Other Oncogenes and Apoptosis.

Other oncogenes, unrelated to the *bcl*-2 family, can also protect against apoptosis and *abl* is of note in this regard. Virtually all chronic myeloid leukaemia (CML) patients are found to have a unique translocation between the *bcr* oncogene loci on chromosome 22 and *abl* on chromosome 9 (Yin, 1995). Expression of chimeric *bcr-abl* prevents apoptosis in CML myeloid precursors, permitting the expansion of the CML leukaemic clone (Bedi, 1994). Other oncogene products acting to modulate apoptosis include c-Myc , p34 and p53 (Amanti, 1993; Marin, 1994; Vaux, 1994). c-Myc is a short lived protein that dimerises with a

long lived heterologous protein - Max, the heterodimer binds to DNA in a sequence specific manner (Marcu, 1992). Myc expression is tightly controlled in non-transformed cells. Mitogen induced c-Myc expression in the presence of appropriate survival factors results in cell cycling, whereas if cells over-express c-Myc or are deprived of survival factors they default to apoptosis (Harrington, 1994). Tissue expression of c-Myc is thus related to the level of cell cycling and the level of expression of c-Myc and degree of apoptosis are correlated (Marcu, 1992).

1. 4. 2. 9 Physiological Relevance of Apoptosis.

Apoptosis is uniquely suited to deletion of cells in normal growth and development, allowing precise control of cell death without invoking pro-inflammatory responses. Apoptosis has been well described in normal embryonal development and metamorphosis (Glucksmann, 1951; Saunders, 1966; Kerr, 1974; Pierce, 1989).

Many hormone sensitive tissues regress by apoptosis in the absence of the appropriate trophic hormone, including the uterus after progesterone withdrawal (Hopwood, 1976; Nawaz, 1987; Rotello, 1989), regression of the lactating mammary gland and prostate epithelial cells (Walker, 1989; Thompson, 1994). Apoptosis also plays an important role in the immune system, selective cell death helping to regulate B and T cells (Liu, 1989; Shi, 1989; Cohen, 1992; Howie, 1994). Activation induced death of immature thymocytes exposed to anti-CD3 has been shown to be by apoptosis in both organ culture and in intact animals (Smith, 1989; Shi, 1991). Furthermore, using transgenic mice, direct evidence is provided that clonal deletion is the result of apoptosis (Murphy, 1990). Mature helper and cytotoxic T cells that have proliferated during an immune

response are cleared by apoptosis; having become IL-2 dependent they die rapidly in its absence (Gillis, 1978; Duke, 1986). Apoptosis is also induced in virally infected cells, tumour cells or allogeneic cells by cytotoxic T lymphocytes (CTL), natural killer (NK) cells and K cells (Duke, 1983; Russell, 1983; Duval, 1986; Zychlinsky, 1991; Cohen, 1992).

Thus the life-span of the cell is tightly controlled, both environmental and genetic influences being able to induce selective death. Any impairment in the controls on cell death can be detrimental, resulting in either excessive cell proliferation or uncontrolled cell death. Pathological states arise where too many cells die such as in central nervous system degenerative diseases or HIV disease where there is inappropriate induction of apoptosis in T cells (Terai, 1991). The converse state exists with neoplasia where the balance between cell proliferation and cell death is shifted resulting in increasing tumour size (Dive, 1991).

1. 4. 2. 10 The Role of Apoptosis in Acute Inflammation.

Apoptosis has an important part to play in control of acute inflammation (Haslett, 1994). The resolution of acute inflammation requires the removal of large numbers of inflammatory cells in a controlled fashion and phagocytic clearance of these apoptotic cells at inflammatory sites in a manner that does not invoke a pro-inflammatory response (Savill, 1989a and b; Grigg, 1991; Haslett, 1994). In *in vitro* culture, PMN (Savill, 1989a) and eosinophils (Stern, 1992) undergo apoptosis constitutively, the rate of which is modulated by cytokines normally present at the inflamed site (Lee, 1993). These apoptotic cells are efficiently phagocytosed by Mø (and fibroblasts) and this phagocytic capacity is also modulated by the cytokine

milieu present (Lee, 1993; Hall, 1994; Ren, 1995); this phagocytosis also occurs at the inflamed site (Savill, 1989a; Grigg, 1991).

The apoptotic process induces specific changes that enables the recognition, binding and phagocytosis of these apoptotic cells by phagocytes (Savill, 1989a and b, 1990 and 1992; Hall, 1994). This utilizes a novel recognition mechanism that does not induce pro-inflammatory responses by the Mø (Meagher, 1992). A model for the recognition mechanism has been proposed, involving CD36 and $\alpha v\beta 3$ - the vitronectin receptor - on the Mø surface, with thrombospondin acting as a molecular bridge to as yet unidentified structure(s) on the apoptotic cell surface (Savill, 1990). It has also been proposed that Mø recognize exposed phosphatidyl serine residues on the apoptotic cells surface (Fadok, 1992a). This alternative recognition system was shown in murine peritoneal Mø and not in murine bone marrow derived Mø or in human monocyte derived Mø, suggesting that there may be alternative recognition mechanisms for different Mø populations (Fadok, 1992b).

1. 4. 2. 11 Monocyte and Macrophage Apoptosis.

During the resolution of inflammation PMN undergo apoptosis and subsequent engulfment by Mø, resulting in their clearance in large numbers in a non-phlogistic manner. The fate of the Mø is however less clear (van Furth, 1992). As discussed in sections 1. 3. 7. 1 and 1. 3. 8 it has been suggested that Mø may die locally in the tissues or migrate to the draining lymph nodes. The relative contribution of these alternative sites for Mø disposal and the mechanism for Mø death *in vivo* are unknown. At the outset of this project there were very few reports on Mø apoptosis (Waring, 1990; Hogquist, 1991a) and only one group had

described monocyte apoptosis (Mangan, 1991a and b). We have now shown that Mø can undergo apoptosis following exposure to cycloheximide (Bellingan, 1994a). A number of other papers have also since emerged showing that Mø are capable of undergoing apoptosis in response to a range of other stimuli including: NO· (Albina, 1993; Sarih, 1993a), silica (Sarih, 1993b), ricin (Khan, 1993), anti-Fas antibody (Iwai, 1994), cross-linking CD69 (Ramirez, 1994), M-CSF depletion (Moore, 1994) and pathogenic organisms including Shigella (Zychlinsky, 1992) and Mycobacteria (Gan, 1995). Despite this, the relevance of this *in vivo* is unknown. The Mø is usually though to be a long lived cell (Rosser, 1970; Melnicoff, 1988b; van Furth, 1992), hence for the inflammatory Mø at the site of resolving inflammation it is not clear whether apoptosis is their normal immediate fate. Indeed, the *in vivo* controls on Mø longevity are poorly understood, many of the demonstrated inducers of Mø apoptosis have been pharmacological rather than physiological agents (Waring, 1990; Khan, 1993; Iwai, 1994; Richardson, 1994). Furthermore, much of the recent experimental work has been on Mø-like cell lines and this may not be relevant to the Mø itself (Cotter, 1992; Zychlinsky, 1992; Kitajima, 1994). There may also be differences in survival characteristics between monocytes and Mø as well as between Mø populations resident in different sites. Certainly the experiments of Mangan and Wahl, showing rapid (<48 hour) monocyte apoptosis in the absence of pro-inflammatory cytokine stimulation (Mangan, 1991 a and b) suggested to me that a difference in the constitutive rates of apoptosis between monocytes and Mø may exist.

1. 4. 2. 12 Mechanisms of Macrophage Apoptosis.

There are some clues as to the mechanisms Mø employ to prevent the induction of apoptosis. Ramirez *et al.* have shown that monocytes exposed to LPS undergo apoptosis when the activation antigen CD 69 (part of the natural killer complex) is cross-linked, or when they are exposed to IL-4. Pre-treatment with pertussis toxin prevents death due to the cross-linking of CD 69 but not that induced by IL-4. This suggests the existence of different apoptosis inducing pathways in the monocyte (Ramirez, 1994). Apo-1 or Fas (CD95) is a 43-kD membrane protein member of the TNF receptor gene family that transmits an apoptosis inducing signal to a variety of cells upon ligand binding (Itoh, 1991; Oehm, 1992). Fas is constitutively expressed on PMN, monocytes, Mø, a proportion of lymphocytes and a subset of endothelial cells. Activated T cells express a functional Fas ligand (Suda, 1993 and 1994) suggesting that T cells could induce Mø apoptosis through Fas ligation (Richardson, 1994). Although Fas ligation induces apoptosis in PMN, only a fraction of Mø are susceptible and Fas ligation has no effect on endothelial cells. It has been suggested that this difference could be explained by differential levels of expression of *bcl*-2 (Iwai, 1994; Richardson, 1994), or by ROI damage (Kohno, 1996) but this remains unproven. Activation induces susceptibility to Fas-mediated apoptosis in B cells (Schattner, 1995) and T cells (Rovere, 1996) and may explain differences in Mø sensitivity to CD95 mediated apoptosis (Richardson, 1994). Mø, like PMN, undergo apoptosis in response to protein synthesis inhibition (Bellingan, 1994a; Khan, 1993; Haslett, 1994). This argues for a continual expression of an inhibitor of apoptosis, depletion of which allows apoptosis to proceed. Interestingly, it has been shown that in the presence of effective protein synthesis inhibition, adherence of Mø prevents apoptosis (Waring, 1990). Long

term Mø cultures can be maintained in serum free medium however, suggesting some degree of resistance to these requirements during differentiation (Darfler, 1982; Helinski, 1988), which may in part be due to paracrine production of survival factors including M-CSF, TNF-α and GM-CSF (Moore, 1994). It has already been suggested that reduced TNF-α binding capacity may explain reduced sensitivity to TNF-α induced apoptosis of bone marrow derived PMN compared with normal PMN (Tsuchida, 1995). As monocytes have been reported to be short lived cells whilst Mø survival is in the order of weeks or months, similar variations in the expression of cytokine receptors and thus responsiveness to cytokine driven pro or anti-apoptotic signals may help to explain these survival characteristics.

1. 5 AIMS.

The aim of this work was to investigate the fate of the Mø and to shed some light on the mechanisms controlling the clearance of Mø from the site of inflammation during resolution. Work described in this thesis will be considered in two broad sections, corresponding to *in vitro* and *in vivo* studies.

The mechanisms and controls on monocyte and Mø apoptosis were investigated *in vitro* using human peripheral blood monocytes, monocyte derived Mø, alveolar Mø, the monocyte-like cell line, U937 and murine bone marrow derived Mø. Reliable systems for the isolation and culture of pure populations of monocytes and Mø were established and a number of independent methods for identifying and quantifying apoptosis in these cells were investigated. To attempt to understand the physiological controls on Mø longevity, the role of a range of cytokines

and influence of changes in expression of cytokine receptors, the effect of serum withdrawal and specific culture conditions, the influence of free radicals and anti-oxidants, as well as the expression of various oncogenes was determined on the induction/prevention of apoptosis in this range of cells.

The fate of the inflammatory Mø during the resolution of inflammation was investigated *in vivo* using a murine model of resolving peritonitis. In this model semi-allogeneic, fluorescent labelled, inflammatory Mø from H-$2^{k/d}$ mice were adoptively transferred into the peritoneal cavity of recipient H-2^{k} mice at the same stage of resolving peritoneal inflammation. Dual colour flow cytometry permitted discrimination between donor cells, recipient cells and donor cells which had been phagocytosed by recipient Mø. Tissue localization and migration kinetics of Mø could be tracked. These studies allowed investigation of the relative contributions of Mø death at the inflamed site with phagocytosis locally and Mø emigration, on the clearance of inflammatory Mø during the resolution of inflammation. They also permitted a comparison of inflammatory and resident Mø emigration kinetics, providing a powerful analytical tool to follow Mø kinetics during resolution of inflammation and open the way to investigating some of the mechanisms controlling the process of resolution.

Chapter 2

MATERIALS AND METHODS.

MATERIALS AND METHODS

2. 1 Reagents.

2. 1. 1 Cell Culture Reagents.

Culture medium including Iscove's modification of Dulbecco's modified Eagles medium (IDMEM), Hank's balanced salt solutions (HBSS) and culture supplements (L-glutamine, HEPES and antibiotics) were purchased from Gibco (Gibco Laboratories, Paisley, Scotland) and sterile tissue cultureware from Falcon (Falcon Plastics, Becton Dickinson, Oxford, U.K.).

2. 1. 2 Chemical and Biological Reagents.

All chemicals were obtained from Sigma (Sigma Chemical Co., Poole, U.K.) unless otherwise stated. Stock solutions of the following were prepared and stored at -20°C: cycloheximide (10 mM), prepared in ethanol; dibutyryladenosine 3'.5'-cyclic monophosphate (db-cAMP) (20 mM in IDMEM); acridine orange (1 mg/ml in IDMEM); dexamethasone (David Bull Laboratories, Warwick, U.K.) (8.2 mM in water, stored at room temperature).

2. 2 Antibodies.

All antibodies and monoclonal antibodies (mAb) were stored at 4°C for short periods and -20°C for long term storage.

2. 2. 1 Antibodies for Indirect Immunofluorescence on Human Cells.

Monoclonal antibodies 38, an IgG2a anti-CD11a and 44, an IgG1 anti-CD11b were both kinds gifts from Dr. N. Hogg, ICRF, London (Dransfield,

1989). The hybridoma clone 3G8, secreting an IgG1 anti-CD16 mAb was the generous gift of Dr. J. Unkless, Mount Sinai Medical School, New York (Fleit, 1982). Anti-Bcl-2 mAb (IgG2a clone 124) and anti-c-myc mAb (IgG2a) were both kind gifts Professor T. Cotter, University College, Cork, Ireland and anti-CD14 IgG2a (clone UCHM1), a gift from Dr. Peter Beverley, University College London (Hogg, 1984). BOB 78, an IgM mAb against an intracellular antigen of unknown specificity was generously supplied by Dr. J. Ross, Lister Laboratories, Edinburgh. Anti-HLA DR, an IgG1 mAb was provided by the Scottish Antibody Production Unit (SAPU, Lau hospital, Carluke, Airshire); anti-CD43, an IgG1 mAb (clone WR-14), was purchased from Serotec (Serotec, Kidlington Oxfordshire), anti-p8,14 mAb from Dako, (Dako, High Wycombe, Bucks.) and anti-BCR-ABL sheep polyclonal antibody from Cambridge Research Biochemicals (Cambridge Research Biochemicals, Northwich, Cheshire). Antibodies against the range of cytokine receptors listed in section 5. 2 were obtained from the Fifth International Workshop on White Cell Differentiation Antigens (Kikutani, 1995). They were supplied at 1 mg/ml purified protein without azide unless stated, the titer for use was suggested by the laboratory of origin in each case, all were murine mAb except C002 and C006, which were rat mAb.

2. 2. 2 Antibodies for Indirect Immunofluorescence on Murine Cells.

The following mAb were used: F4/80 (Austyn, 1981), an IgG2b murine Mø specific mAb, produced by the HB 198 clone (purchased from American Type Culture Collection (ATCC), Rockville, MD; M1/70 an IgG2b rat mAb to murine Mac-1 (CD11b) expressed on Mø and PMN (Serotec); MRC OX-78 an anti-CD48 IgG2a rat mAb to murine CD48,

binding B cells, T cells and Mø (Serotec); 7/4 a rat mAb to a polymorphic antigen on murine PMN (Serotec); MRC OX-6, a mouse anti-mouse Ia IgG1 mAb to dendritic cells, B lymphocytes and some Mø (Serotec). The clone Mac 51, secreting an IgG2b rat anti-potato cytochrome mAb was used as a negative control (purchased from European Collection of Animal Cell Cultures (ECACC), Porton Down Wilts.); the hybridoma MOPC-21C secreting a non-binding murine IgG1 mAb (purchased from ECACC) was also used as a negative control.

2. 2. 3 Directly Conjugated Antibodies (Human).

A direct fluorescein isothiocyanate (FITC)-conjugated anti-myeloperoxidase mAb (Serotec), and a FITC-conjugated 3G8 anti-CD16 mAb (kind gift Dr. I. Dransfield, University of Edinburgh) were used as indicated.

2. 2. 4 Directly Conjugated Antibodies (Murine).

FITC-conjugated mouse anti-mouse H-2D^{d} IgM mAb (Pharmingen, San Diego, CA); FITC-conjugated mouse anti-human CD15 IgM mAb (Dako) as control; FITC-conjugated mouse anti-mouse H-2K^{k} IgG_{2a} mAb (Pharmingen); FITC-conjugated mouse anti-human T cell CD45RO IgG_{2a} mAb (Dako) as control; MRC OX-6, (MCA 46F) a FITC conjugated mouse anti-mouse Ia IgG_1 mAb (Serotec) was used to determine the level of Class II expression.

2. 2. 5 Secondary Antibodies for Indirect Immunofluorescence.

FITC-conjugated $F(ab')_2$ rabbit anti-mouse (Dako); FITC-conjugated donkey anti-sheep (Serotec); FITC-conjugated $F(ab')_2$ rabbit anti-rat (Serotec). Phycoerythrin (PE)-conjugated $F(ab')_2$ rabbit anti-mouse (Dako).

2. 3 Isolation and Culture of Human Peripheral Blood Monocytes.

2. 3. 1 Purification of Mononuclear Cells by Discontinuous Percoll Gradient Centrifugation.

All purification procedures were performed under sterile conditions, using sterile reagents and plasticware in a class II hood. Mononuclear cells were prepared by the methods described by Dooley *et al.* (Dooley, 1981) and modified according to Haslett *et al.* (Haslett, 1985). Briefly blood was obtained under sterile conditions from a pool of normal healthy volunteer male and female donors, with an age range of 20 -55 years. Venous blood (36 mls/tube) was drawn into 50 ml polyethylene tubes, mixed with 4 mls/tube of 3.8% w/v sodium citrate, each donor providing 6 tubes (216 mls of blood). The citrated blood was centrifuged at 300 x g for 20 minutes at room temperature in a MSE Mistral 3000 centrifuge (Sanyo Gallenkamp, Loughborough, U.K.). Platelet rich plasma (PRP) supernatant was aspirated and autologous serum and platelet poor plasma prepared from this by centrifuging a portion of the PRP at 2500 x g for 20 minutes to prepare platelet poor plasma supernatant, serum was prepared from the remainder by recalcification of 10 mls PRP/glass tube with 220 µl 0.32% calcium chloride solution and incubating at 37°C for 30 minutes. The blood cell pellet was meanwhile mixed gently with 5 mls of 6% dextran (500 000 m. w.) (Pharmacia Fine Chemicals, Piscataway N.J.), the volume corrected to 50 mls with 0.9% w/v saline at 37°C and sedimentation of erythrocytes allowed to proceed at room temperature for 30 minutes. After dextran sedimentation the leukocyte enriched upper layer was aspirated and washed with normal saline. A discontinuous Percoll (Pharmacia) gradient was prepared from a stock solution of Percoll containing 9 parts Percoll and 1 part 10 x

Phosphate Buffered Saline (10 x PBS) (thus isotonic). Percoll of different percentages (70% and 81%) were prepared by diluting stock Percoll with appropriate quantities of Hank's balanced salt solution without calcium and magnesium (HBSS w/o) and the gradients produced by carefully overlayering 3 mls 70% Percoll upon 3 mls 81% Percoll in 15 ml tubes (1 tube per 80 mls of citrated blood). The cell pellet after dextran sedimentation was mixed with 55% Percoll, layered over the 70% Percoll and centrifuged at 700 x g for 20 minutes at room temperature. The mononuclear cells were aspirated from the 55/70% interface, granulocytes from the 70/81% interface and erythrocytes were pelleted at the bottom of the tube. Cells were washed 3 times, twice in HBSS w/o and finally in HBSS, counted on a Neubauer haemocyometer. Viability was assessed by determining the proportion of cells which were able to exclude the vital dye, trypan blue. Viability was routinely >98% and preparations with viabilities less than 98% were discarded. Cells were then resuspended as required.

2. 3. 2 Purification of Monocytes from Mononuclear Cells.

2. 3. 2. 1 Plasma Plate Adherence.

Monocytes were separated from the more numerous lymphocytes on the basis of adherence, using minor adaptions of well established methods (Ackerman, 1978). Briefly, 140 mM tissue culture dishes were coated with plasma by diluting autologous platelet poor plasma 1:10 with 1x PBS and adding 3 mls/plate. These plates were incubated for 1 hour at 37°C then washed twice with PBS. Mononuclear cells were resuspended at 4 x 10^6/ml in IDMEM with 10% autologous serum and 7 mls added per plate. Plates were incubated at 37°C in 5% CO_2 for 1 hour and the non-adherent cells washed off with HBSS w/o, using sterile transfer pipettes. In order

to detach adherent monocytes 3 mls of pre-warmed 5 mM ethylene diamine tetra-acetic acid (EDTA) (BDH, Glasgow, U.K.) in PBS containing 20% autologous serum was added per plate for 10 minutes at 37°C. Cells were rinsed off vigorously with IDMEM and washed twice again in IDMEM containing 0.1% gelatin before culture. Yields of between 10 and 30 x 10^6 monocytes/216 mls of blood were usually achieved, with a purity of 90.2% (range 84.6 to 96%, n = 81), assessed by cytospin morphology and flow cytometry (section 3. 2. 1. a).

2. 3. 2. 2 Tissue Culture Plastic Adherence.

Washed mononuclear cells from the discontinuous Percoll gradient were suspended in IDMEM at 4 x 10^6/ml and allowed to adhere for 1 hour to either 24 or 96 well tissue culture plates (1 ml/well for 24 well plates or 150 µl/well for 96 well plates) at 37°C in 5% CO_2 in a humidified incubator (standard conditions). The non-adherent cells were then removed by vigorous and repeated washing with HBSS w/o using a sterile transfer pipettes. The remaining adherent monocyte were cultured on *in situ* under standard conditions.

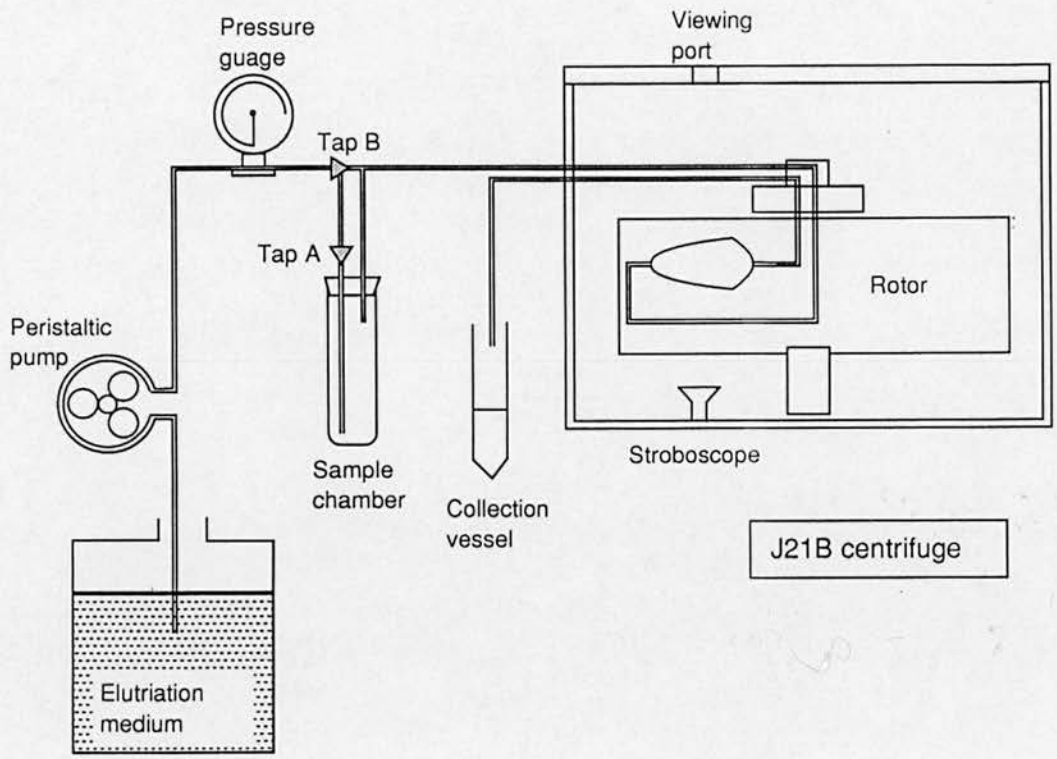
2. 3. 2. 3 Purification of Monocytes using Counterflow Centrifugal Elutriation (CCE).

Monocytes, granulocytes and lymphocytes can be prepared using CCE, which separates cells on the basis of their differential sedimentation velocities (Figdor, 1983). A schematic diagram of an elutriator is shown in **Figure 2. 1**. Sedimentation velocity is determined by several factors including cell density, size ,shape and cell membrane characteristics allowing accurate separation of cells. During elutriation the centrifugal force, provided by the centrifuge, is balanced by the inward flow of

medium (IDMEM plus 0.2% autologous serum) pumped by a variable speed peristaltic pump. Cell suspensions are maintained in the elutriation chamber and then by increasing the pump speed (or decreasing the centrifugal force), cells of increasing sedimentation velocity can be separated, normally with good yields and minimal loss of viability (Wahl, 1984).

A J-21 Beckman centrifuge fitted with a JE-6B elutriation rotor was used along with a Masterflex peristaltic pump (both supplied by Beckman Instruments, High Wycombe, U.K.). The elutriator was cleaned and sterilized with 100% ethanol and distilled water then primed with medium, ensuring all bubbles were removed and the flow rate calibrated by varying the pump speed. The cell pellet after dextran sedimentation was washed, resuspended in 5 mls HBSS w/o and injected into the CCE sample chamber using tap A, with the by-pass tap B switched to by-pass the sample chamber. Cells were then loaded into the elutriation chamber by switching tap B to pump medium through the sample chamber (see **Figure 2. 1**). Initially a flow rate of 18 mls/minute and a rotor speed of 2500 r.p.m. was used to elute off erythrocytes, this was increased to 21 mls/minute for lymphocytes and 24 to 27 mls/minute for monocytes; precise flow rates varying with each separation. Cells were collected in 50 ml fractions and purity assessed by flow cytometry before significant adjustments were made to the pump speed. PMN were finally eluted out by increasing the pump speed to 35 mls/minute. Yields of between 15 and 44 x 10^6 monocytes/216 mls of blood were obtained, with a purity of 91.2% (range 80 to 96%, n = 31 experiments).

Figure 2. 1 Schematic Diagram of an Elutriator.



Legend for Figure 2. 1 Schematic diagram of an elutriator showing how medium was drawn from a reservoir into the elutriation chamber which lay within the rotor arm of the elutriator. To introduce cells into the elutriator, tap B was switched to allow the flow of medium to by-pass the sample chamber and the cells were injected into the sample chamber using tap A. Cells were then loaded into the elutriation chamber by switching tap B to pump medium through the sample chamber. The flow rate of the variable speed peristaltic pump was then steadily increased and cells were collected in 50 ml fractions.

2. 4 Maturation of Monocytes into Macrophages.

Three methods of monocyte culture were employed; for all experiments culture medium consisted of IDMEM containing 10% autologous serum, 100 units/ml penicillin and 100 μg/ml streptomycin (standard culture media unless otherwise stated) and cells were cultured under standard conditions.

2. 4. 1 Suspension Culture in Hydrophobic Teflon Trays.

Teflon foils of varying sizes from 1 to 5 cm square and 1 cm deep were made by sealing the edges of Teflon fluorinated ethylene propylene sheets (Teflon; ChemFab, Handforth, Cheshire, U.K.) using a modified HM3000 Impulse heat sealer (Hume Martin Ltd., London, U.K.) and autoclaved prior to use. These foils could hold between 1 and 12 mls of medium. Monocytes were washed twice in IDMEM containing 0.1% gelatin, resuspended at 1 x 10^6/ml in standard culture medium and placed in a Teflon foil of appropriate size. These were seated in a covered 140 m M petri dish and incubated for 5 days under standard conditions. For certain experiments autologous serum was withheld, an equal volume of IDMEM being added to maintain the final concentration at 1 x 10^6 cells/ml. Suspension culture yielded Mø with a purity of 92.9% (range 85.6 - 98%, n = 73 experiments) and a viability consistently >99%.

2. 4. 2 Adherent Culture on Tissue Culture Plastic.

Adherent monocytes in 24 or 96 well plates (section 2. 3. 2. 2) were covered with 1 ml or 150 μl respectively of culture medium and incubated. The medium was replaced after 3 days, non-adherent cells being washed off and fresh culture medium replaced. Adherent Mø populations of 96 to 98% purity were obtained.

2. 4. 3 Adherent Culture in 8 Well Slides.

Mononuclear cells were resuspended at 1 x 10^6 cells/ml in IDMEM and 170 µl/well added to 8 well detachable tissue culture slides (Nunc - supplied by Falcon Plastics), this was made to a final volume of 200 µl/well with the addition of autologous serum, test reagents or further IDMEM, as described for each experiment. Slides were covered and incubated under standard conditions until used. The percent apoptosis and the number of cells remaining in each treatment well was assessed at 6, 18 and 42 hours and at 5 days, separate slides being prepared for each time point. Paired experiments were performed on Mø from the same donors, these being matured in Teflon foils for 5 days, washed twice in IDMEM containing 0.1% gelatin and placed in wells as described for monocytes and assessed again at 6, 18 and 42 hours.

2. 5 Isolation and Culture of Other Leukocytes.

2. 5. 1 Preparation of Human Polymorphonuclear Leukocytes.

PMN were prepared either by discontinuous Percoll gradient centrifugation by collecting cells at the 81% interface (section 2. 3. 1) or by CCE at pump speeds of 35 mls/min. (section 2. 3. 2. 3). Cells were washed twice in IDMEM containing 0.1% gelatin and resuspended in culture medium. Cell preparations were >99% viable as assessed by the ability to exclude the vital dye trypan blue and were >97% pure as assessed by Dif Quik™ stained cytospin preparations. For aging overnight to induce apoptosis the cells were placed in 75 cm^2 tissue culture flasks at 4 x 10^6/ml with 10% autologous serum at 37°C in 5% CO_2. In experiments to assess the effect of ROI or anti-oxidants on PMN apoptosis, 2% rather than 10% autologous serum was used and cells were resuspended at a final concentration of 1 x 10^6 cells/ml after addition of test reagents and at a

final volume of 400 µl per well in 8 well slides, these were covered and incubated under standard conditions for 8 to 24 hours.

2. 5. 2 Preparation of Human Lymphocytes.

Lymphocytes were obtained from the non-adherent cells following the plasma plate adherence step of the monocyte preparation (section 2. 3. 2. 1). These cells were re-adhered to plasma coated plates to further reduce the number of contaminating monocytes and the non-adherent cells from this second adherence step were collected, yielding a population containing >89% lymphocytes as assessed by flow cytometry and 87% as assessed by Dif Quik™ stained cytospin preparations. The lymphocytes were placed in culture medium at 1 x 10^6 cells/ml in Teflon foils and cultured under standard conditions until used.

2. 5. 3 Human Alveolar Macrophages.

From time to time human alveolar Mø became available following bronchoalveolar lavage of normal volunteers, all of whom had abstained from smoking for at least 12 hours. Lavage was performed by Dr. D. Morrison and Dr. W. MacNee (Respiratory Medicine Unit, Royal Infirmary, Edinburgh, in standard fashion under local anaesthesia using 150 mls of PBS as the lavageate. Cells were strained through sterile gauze, washed and resuspended at 1 x10^6/ml in IDMEM with penicillin, streptomycin with or without 10% heat inactivated human AB serum (Sigma). Heat inactivated serum was prepared by heating pooled human AB serum (Sigma) to 56°C for 30 minutes and allowing it to cool before filtering through 0.45 µm filters (Millipore, Watford U.K.) and storing at -20°C until used. Cells were placed in Teflon foils and incubated.

2. 5. 4 Preparation of Murine Bone Marrow Derived Macrophages.

2. 5. 4. 1 Preparation of CSF-1 Containing Medium.

L929 cells, a murine fibroblastoid cell line known to secrete CSF-1 (Akagawa, 1988), were obtained from ECACC (Catalogue No. 85011425) and grown to confluence over 7 days in 175 cm^2 tissue culture flasks using IDMEM containing penicillin, streptomycin and 10% Foetal Calf Serum (FCS) (Life Technologies, Paisley, U.K.). The supernatant was harvested, cleared of cells by centrifugation at 2500 x g and filtered through 0.22 µm filters (Millipore) and stored frozen at -70°C until required.

2. 5. 4. 2 Isolation and Culture of Murine Bone Marrow Derived Macrophages.

Outbred BKW mice 8-12 weeks old were killed by cervical dislocation, both femurs were dissected, cleaned of muscle and connective tissue and the epiphyses cut off exposing the medullary cavity from which the marrow was flushed out using a 25 gauge needle and 2 mls standard murine culture medium (IDMEM, 25% FCS, 25% L929 cell supernatant with addition of penicillin (100 U/ml) and streptomycin (100µg/ml)and HEPES buffer to a final concentration of 25 mM) (van der Meer, 1983). Cell clumps were disrupted and cells washed, counted and seeded into Teflon foils at 0.25 - 0.5 x 10^6 cells/ml in standard murine culture medium. After 5 days culture under standard conditions cells were transferred into fresh medium and were used on day 7 to 9 (Tushinski, 1982). To demonstrate the Mø morphology, surface marker expression and functional characteristics of these cells the following tests were performed. Cytospin preparations stained with Dif Quik™ demonstrated uniform cell morphology with large slightly vacuolated appearance and a

single large nucleus typical of Mø, the cells all stained positive (although weakly) for non-specific esterase. Antibody labelling confirmed that they were positive for F4/80 and Mac 1 and weakly positive for CD48. Flow cytometry confirmed the cells were a single population with forward and side scatter characteristics similar to those of murine peritoneal Mø. Functional studies demonstrated the cells were capable of ingesting opsonized zymosan and apoptotic human PMN.

2. 6 U937 Cell Culture.

Cells from the myelomonocytic U937 cell line (ECACC Catalogue No 87010802), derived from a human histiocytic lymphoma, were cultured at 1 x 10^6 cells/ml in 75 cm^2 tissue culture flasks in standard U937 cell culture medium (RPMI plus 10% FCS, penicillin, streptomycin and 2 mM L-glutamine). They were passaged on alternate days, washed and resuspended at approximately 1 x 10^6 cells/ml prior to use. In certain experiments serum was omitted or IDMEM rather than RPMI was used.

2. 7 Assessment of Purity of Monocytes and Macrophages.

2. 7. 1 Cytospin Preparations: Dif Quik™ and Non-specific Esterase Staining.

Cytospin preparations were made using a Shandon Cytocentrifuge (Shandon, Pittsburgh, PA.). Approximately 0.2 x 10^6 cells in 100 µl medium containing 10% serum were added per cytospin chamber, centrifuged onto glass microscope slides at 300 r.p.m. for 3 minutes, air dried, fixed in methanol and stained. Routine staining was with Dif Quik™ stains (Baxter, Thetford, Norfolk) after which a coverslip was applied and cells examined by light microscopy at 400 x magnification. To determine the proportion of monocytes or Mø in a cell population, 400

cells were examined per slide. Selected slides were stained for non-specific esterase according to the methods of Yam *et al.* (Yam, 1980; Shibata, 1985). Briefly, slides were fixed in ice cold formalin acetone (pH 6.6), washed with distilled tap water and air dried. Pararosaniline hydrochloride was neutralized using 5 molar sodium hydroxide and was then hexazoatized by incubation with fresh sodium nitrite (4%) for 1 minute. This was added to sodium phosphate buffer, then α-naphthyl acetate, dissolved in ethylene glycol mono-methyl ether was added giving a white precipitate. The solution was filtered and slides incubated in the filtrate for 45 minutes at 37°C then washed thoroughly and counterstained with methyl green (1% w/v). Microscopic examination revealed characteristic brown staining with non-specific esterase activity.

2. 7. 2 Flow Cytometry.

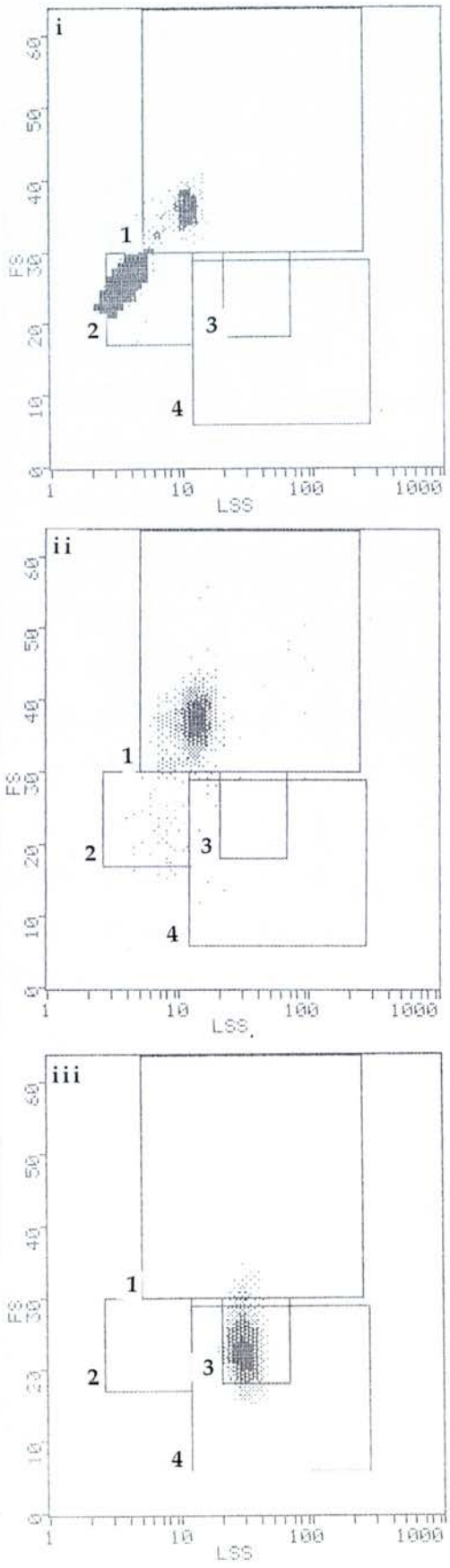
2. 7. 2. 1 Identification of Cell Populations using "Size and Granularity" Criteria.

Flow cytometry was performed using a Coulter EPICS II^{TM} flow cytometer (Coulter Electronics, Luton, U.K.) and cell sorting was performed using a Becton Dickinson FACStar Plus cell sorter (Becton Dickinson U.K.). The optical alignment of the flow cytometer was optimized prior to each run using Immunocheck fluorospheres (Coulter). Different cells have distinct forward and side scatter characteristics, allowing "gates" to be created for the following:

Gate 1	Monocyte/Macrophages	Mø Gate
Gate 2	Lymphocytes	Lymphocyte Gate
Gate 3	Polymorphonuclear leukocytes	PMN Gate
Gate 4	Apoptotic Macrophages	Apoptotic Mø Gate

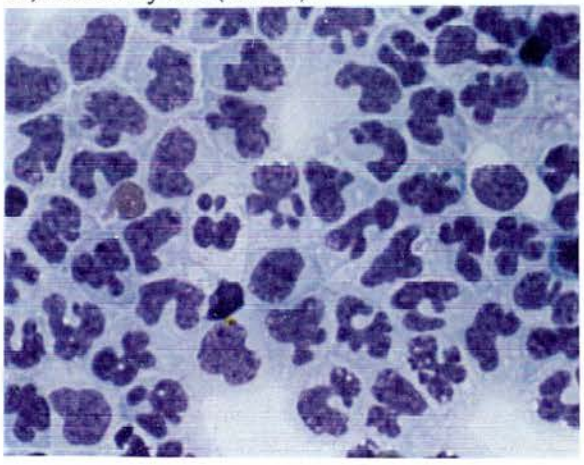
(see validation in results section 3. 2. 8)

Figure 2. 2 Identification of Leukocytes by Flow Cytometry using Forward and Side Scatter.

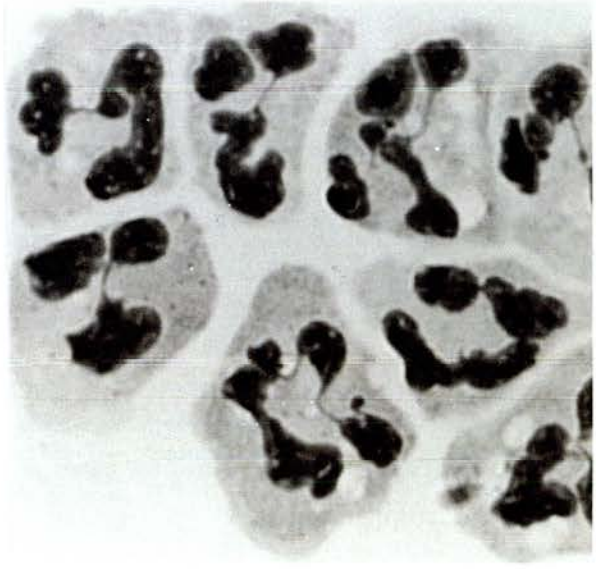


"Gates" were created as follows: 1) Mø Gate, 2) Lymphocyte Gate, 3) PMN Gate, 4) Apoptotic Mø Gate. Pure monocytes, lymphocytes, PMN and mononuclear cells were used to verify the gates, as shown for i) mononuclear cells from Percoll preparation, ii) monocytes and iii) PMN from CCE run. Corresponding cytospins are shown for ii) and iii).

ii) Monocytes (x 200).



iii) PMN (x 400).



The first three gates were validated by running populations with known ratios of cells, determined from cytospin stained preparations. **Figure 2. 2** shows representative cytospin preparations of monocyte and PMN elutriation fractions and the corresponding flow cytometric results. Indirect immunofluorescence analysis using specific antibodies for each cell type and back-gating onto the forward/side scatter plot confirmed these gates were appropriate. The advantage of this method of cell assessment was both its rapidity and accuracy with 5000 cells being counted per assay. Non-apoptotic and apoptotic Mø could also be distinguished on the basis of forward and side scatter (section 3. 2. 8 and **Figure 3. 7**), allowing surface receptor characteristics of these two populations to be determined.

2. 7. 2. 2 Immunolabelling of Cells.

2. 7. 2. 2a Direct and Indirect Immunofluorescence of Intact Cells.

Both direct and indirect cell labelling was performed at 4°C using pre-cooled materials. For immunolabelling of murine peritoneal Mø, cells were blocked with normal mouse serum (NMS) for 15 minutes at 4°C prior to each step and all antibodies were diluted in NMS. Cells were suspended at 2 x 10^6 cells/ml and 150 µl placed into relevant wells of 96 well flexible assay plates supported in 96 well tissue culture plates (Costar, High Wycombe, Bucks.). All cells were washed twice with Tris buffered saline containing 0.2% bovine serum albumin (BSA) and 0.1% sodium azide (pH 7.4), resuspended in 25 µl of saturating concentrations of primary antibody solution and incubated for 30 minutes on ice. After a further three washes cells were resuspended in 25 µl of appropriate FITC-conjugated second layer antibody, incubated for 30 minutes, washed three

times and fixed in PBS containing 0.5% formaldehyde. For direct immunofluorescence the primary antibody was directly conjugated to a fluorescent marker, hence the second incubation was omitted. Negative controls for direct immunofluorescence were provided by directly conjugated non-binding antibodies. In all cases cells were only designated as having positive binding when the fluorescence intensity was greater than that for the isotype control non-binding antibody. Analysis of fluorescence was done on a 4 decade log scale and reported as i) percent positive; indicating the percentage of the population with a fluorescent intensity greater than that of the control antibody and ii) relative mean fluorescence whereby the mean channel fluorescence for the test population was divided by that of the isotype matched non-binding control antibody.

2. 7. 2. 2b Immunofluorescence of Permeabilised Cells.

The intracellular levels of protein product of the following oncogenes were determined: *bcl-2, c-myc* and *bcr-abl.* To confirm that the antibodies were binding, HL-60 cells (kind gift of Dr. S. Hannah, University of Edinburgh) were permeabilised and used as positive controls for the anti-Bcl-2 antibody and fibroblast primary cultures (kind gift of Dr. L. Biddlestone, University of Edinburgh) for the anti-c-Myc antibody. Use of non-permeabilised cells confirmed that the oncogenes were indeed intracellular proteins whilst an antibody against the intracellular antigen p8,14 was included as a positive control and MOPC-21C as a negative control, sheep serum was used as control for the sheep polyclonal anti-Abl antibody (where a FITC conjugated donkey anti-sheep antibody (Serotec) was used for the second layer). A direct conjugate FITC-anti-myeloperoxidase antibody was also employed, reduction in

expression confirming monocyte maturation into Mø. Cells were placed in a 96 well plate at 3 $x10^5$ cells per well, washed and permeabilised by adding 50 µl of permeabilising solution for 15 minutes at 37°C (permeabilising solution final concentrations; Lysophosphatidylcholine 0.01% w/v Formaldehyde (BDH) 3.7% v/v made up with PBS). Cells were washed twice in Tris buffered saline containing 0.4% BSA, the primary antibody added for 30 minutes at 4°C, washed twice, the FITC conjugated second layer antibody then added for 30 minutes and washed twice more before flow cytometric analysis.

2. 7. 2. 3 Dual Colour Immunofluorescence.

2. 7. 2. 3a Apoptosis Induced Changes in Neutrophil Cytokine Receptor Expression.

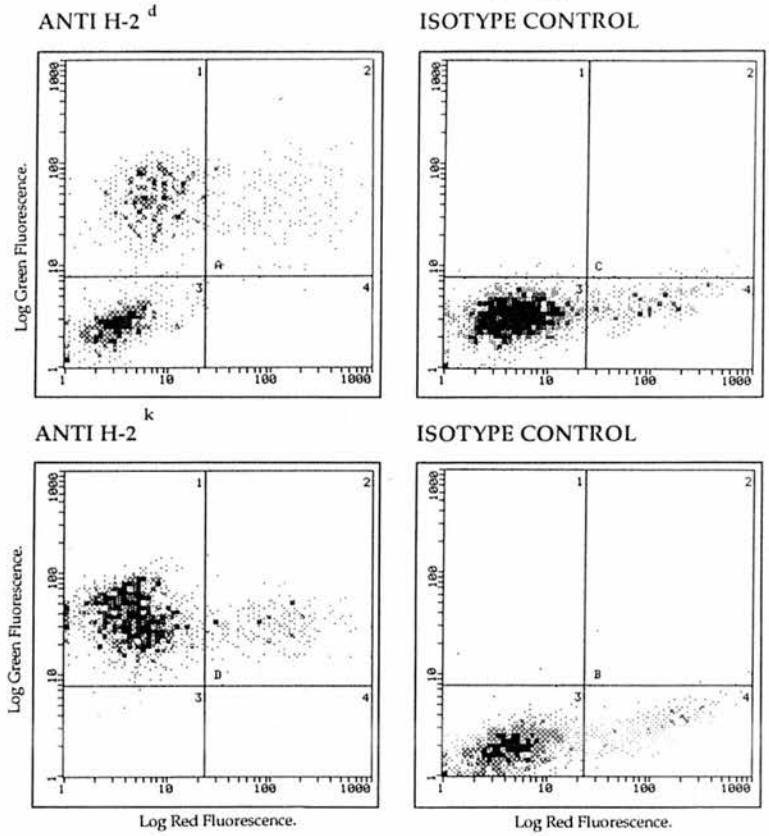
Changes in human PMN cytokine receptor expression with apoptosis were determined using dual colour immunofluorescence, taking advantage of the fact that apoptotic PMN lose expression of the antigen CD16 (Dransfield, 1994). Cells were incubated with anti-cytokine receptor antibodies, washed, incubated with PE conjugated $F(ab')_2$ rabbit anti-mouse (Dako) as the second layer, washed again and incubated with FITC-conjugated anti-CD16 mAb (FITC-3G8) before final washing. To compensate for any signal recorded as red fluorescence from a green signal and vice versa, specific controls were run. First a double negative with no added first layer or FITC-3G8 was run and then a green positive control with FITC-3G8 added but no PE anti-mouse used. Any red signal from this green control was electronically "removed", likewise a red only positive control with anti-CD43 (Serotec) then PE anti-mouse but no FITC-3G8 was run which allowed electronic compensation to be made for any red signal cross-over into the green fluorescence. MOPC-21C was

used as an IgG1 isotype non-binding control with PE anti-mouse and FITC-3G8. To control for any uptake of antibody due to loss of membrane integrity, the antibody BOB78 which binds to an unknown intra-cytoplasmic antigen in PMN was used (I. Dransfield, personal communication). Non-apoptotic and apoptotic PMN were thus distinguished on the basis of their CD16 expression (green fluorescence) and gating on these two populations, their expression of cytokine receptors could be determined using red fluorescence by their binding of PE conjugated second layer.

2. 7. 2. 3b Quantification of Macrophage Phagocytosis *In Vivo* During Resolution of Acute Inflammation.

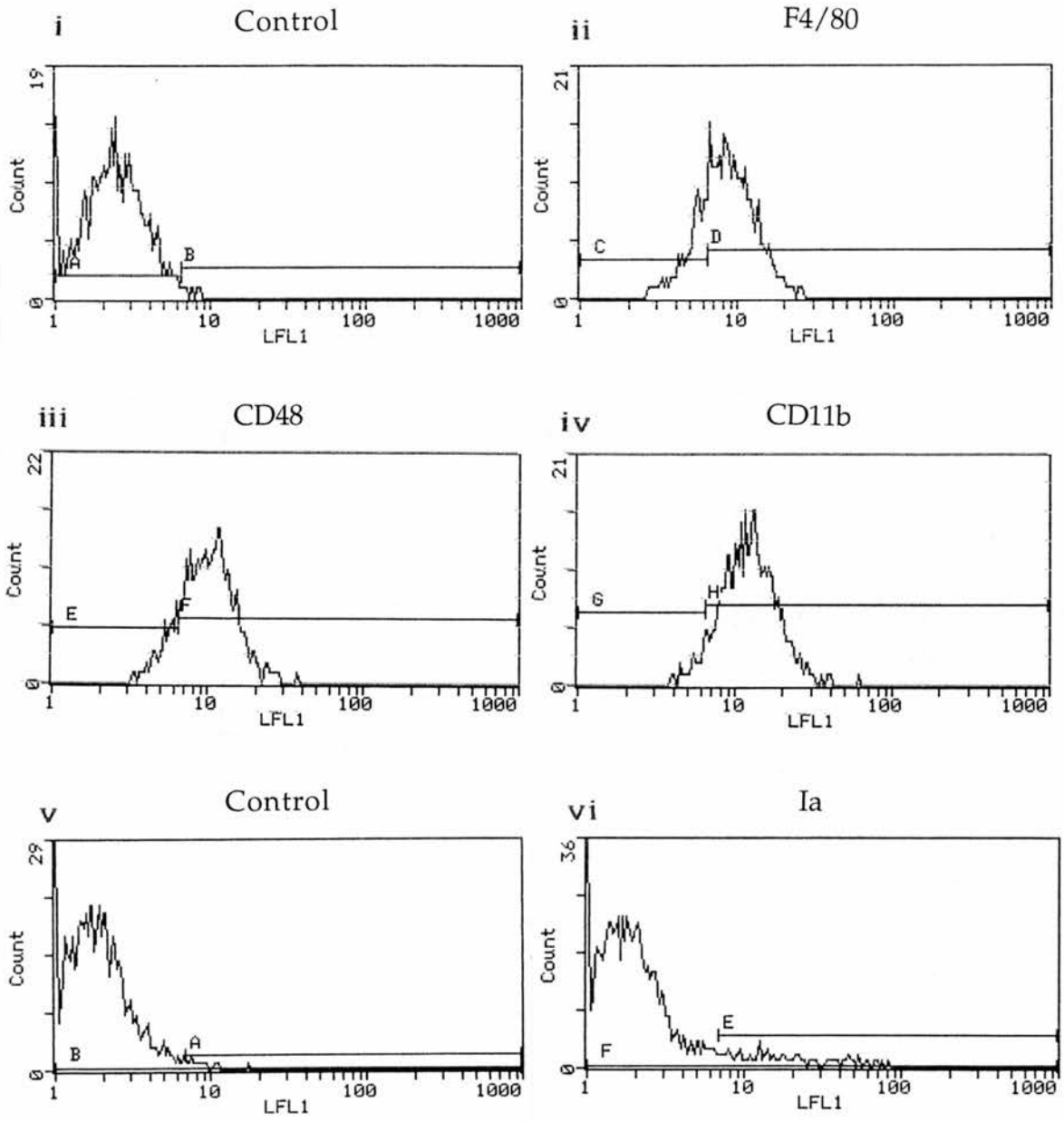
The degree of phagocytosis of donor murine peritoneal Mø by recipient Mø *in vivo* was quantified using dual colour immunofluorescence. Donor Mø expressed H-$2^{k/d}$ whilst recipient Mø expressed H-2^{k} only. Approximately one third of the donor Mø were also labelled with the red fluorescent cell tracking dye PKH26-PCL (section 2. 9. 3. 1b). Donor and recipient Mø could be distinguished by expression of H-2^{d}, detected with a direct FITC-conjugated anti-d mAb, furthermore recipient cells that had phagocytosed donor Mø would gain red PKH26-PCL fluorescence but remain anti-d negative (summarized in **Figure 2. 3**). Using highly purified cells (>94% Mø) obtained by adherence preparation from BHK coated plates (section 2. 9. 5. 1) the forward and side scatter characteristics of inflammatory Mø were determined and a gate on Mø set. This was confirmed by indirect immunofluorescence using the following mAb: F4/80, M1/70 (anti-CD11b), MRC OX-78 (anti-CD48), with Mac 51 (anti-potato cytochrome) as the control, shown in (**Figure 2. 4a and b**).

Figure 2. 3 Use of Dual Colour Immunofluorescence to Distinguish Donor Macrophages, Recipient Macrophages and Phagocytosed Donor Macrophages.



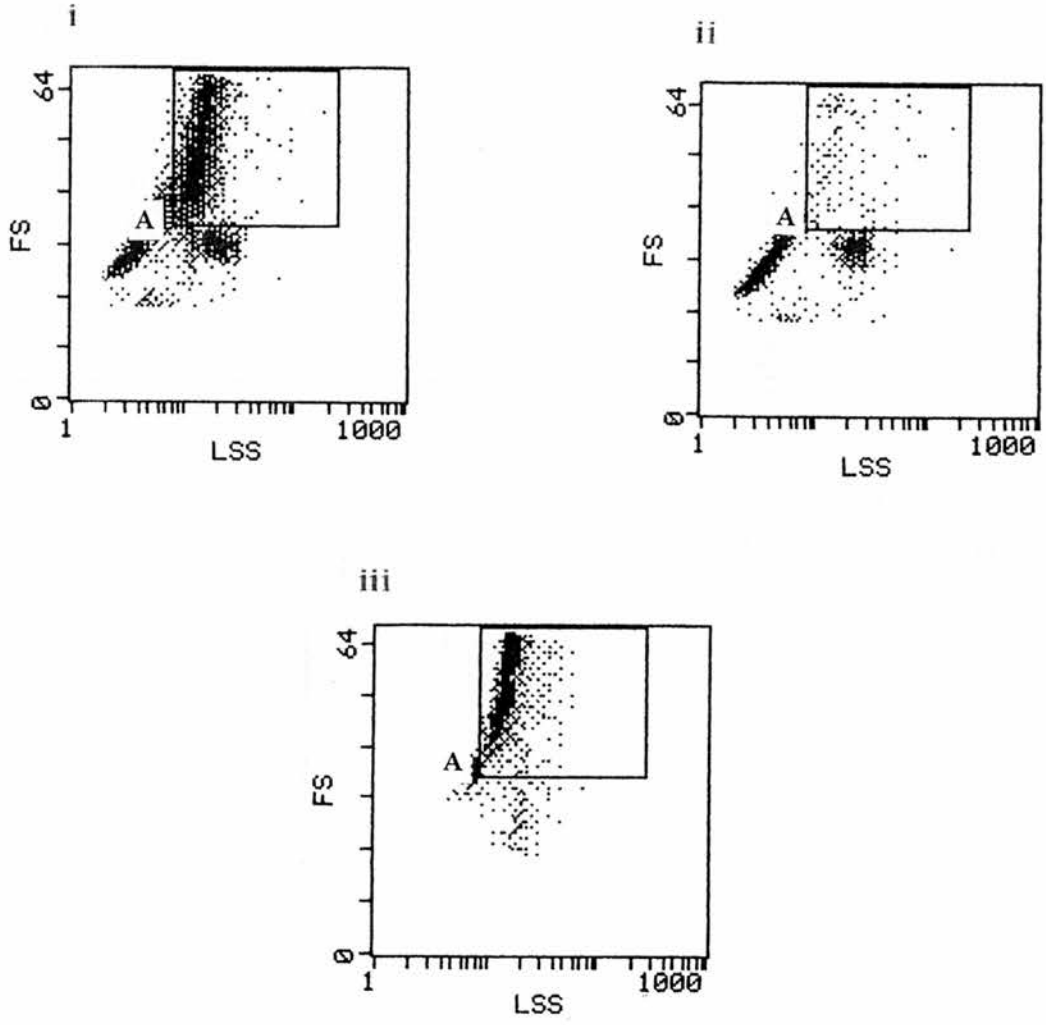
Legend for Figure 2. 3 Representative experiment showing two colour flow cytometry of Mø from peritoneal lavage of a C3H recipient mouse (H-2^k), 48 hours after adoptive transfer of 30 x 10^6 live cells from a CB1 (H-$2^{k/d}$) donor mouse, one third of which were PKH26-PCL labelled. Green fluorescence (FITC-conjugated mAb) on the y axis and red fluorescence (PKH26-PCL) on the x axis. Binding of anti-H-2^d and H-2^k mAb and their isotype controls are shown. Four regions are identified for each mAb: 1 = green positive, red negative, 2 = green positive, red positive, 3 = green negative, red negative and 4 = green negative, red positive. PKH26-PCL labelled cells were thus [anti-k region 2 - isotype control region 2], H-2^d positive cells were [anti-d region 1 and 2 - isotype control region 1 and 2] and phagocytosed cells [anti-d region 4 - anti-k region 4].

Figure 2. 4a Use of Indirect Immunofluorescence to Confirm Purity of Murine Inflammatory Macrophage Preparations.



Legend for Figure 2. 4 Indirect immunofluorescence of murine inflammatory Mø, showing binding of the following mAb **i)** isotype control mAb (for **ii - iv**), **ii)** mAb F4/80, **iii)** MRC OX-78; anti-CD48, **iv)** M1/70; anti-CD11b, **v)** isotype control mAb for (**vi**) and **vi**) MRC OX-6, anti-Ia. This demonstrates little Ia expression (<10% of cells) but all Mø express CD11b, CD48 and bind mAb F4/80.

Figure 2. 4b Use of Flow Cytometry to Confirm Purity of Murine Inflammatory Macrophage Preparations.



Legend for Figure 2. 4b Forward and side scatter profiles of inflammatory Mø from peritoneal lavage of mice five days after i.p. TG injection are shown in **i**). Using back-gating from CD11b, F4/80, CD48 and Ia profiles as shown in Figure 2. 4a, the gate A was set for Mø, confirming that 85% of cells in this preparation were Mø. Mø were further purified by adherence to BHK coated plates, the flow cytometry profiles of the non-adherent cells washed off are shown in **ii**) and the adherent Mø were then lifted off using EDTA, their profile is shown in **iii**) where 95.8% of cells were Mø.

The red and green fluorescence parameters of these Mø could then be determined by dual colour flow cytometry; cells from peritoneal lavageates were examined for expression of PKH26 (red fluorescence) and H-2^d, H-2^k or Mø surface markers (green fluorescence). Anti-H-2^d and H-2^k binding was expressed relative to isotype control direct conjugate mAb and PKH26 expressed relative to non-labelled Mø. Using quad-stats, gated on Mø, 4 regions could thus be identified for each antibody: 1 = green positive, red negative, 2 = green positive, red positive, 3 = green negative, red negative and 4 = green negative, red positive. PKH26 fluorescent red positive cells were thus [anti-k region 2 - isotype control region 2] whilst H-2^d positive cells were [anti-d region 1 and 2 - isotype control region 1 and 2]. Phagocytosed cells were [anti-d region 4 - anti-k region 4], (**Figure 2. 3**).

2. 7. 2. 4 Cell Sorting.

Cells were sorted at a rate of 200 000 cells/hour on the basis of size determined by forward and right angle light scatter and collected into ice cold IDMEM containing 10% autologous serum to minimize further changes.

2. 7. 3 Induction of Apoptosis in Cultured Cells: Effect of Addition or Withdrawal of Agents.

2. 7. 3. 1 Serum Withdrawal.

Serum was withdrawn from cells in suspension culture by washing three times in IDMEM with the addition of 0.1% gelatin to maintain cell viability during recovery. Adherent Mø were simply washed three times with IDMEM to remove serum. Yield and percentage apoptosis was determined at 6, 18 and 42 hours.

2. 7. 3. 2 Cytokines and Other Agents.

All cytokines were purchased from Genzyme, (Genzyme, West Malling, U.K.), the following were tested: recombinant human TNF-α, recombinant human IL-1α, recombinant human IL-4 for human cells and recombinant murine IL-4 for murine Mø, recombinant human IL-6, recombinant human GM-CSF and ultrapure natural human TGF-β. Lyophilized cytokines were diluted with culture medium containing 1% BSA as per manufacturers instructions and stored at -70°C until use (within 6 months of purchase). Cytokines were diluted from stock prior to addition to cell cultures such that a final volume of 5 µl was added per ml of cells, control cells had 5 µl medium plus 1% BSA added. In collaboration with other members of the laboratory, cytokine activity from each batch was confirmed by testing on other cell types and showing activity as expected according to published data: GM-CSF (50 units/ml) and IL-1α (1000 units/ml) significantly inhibited PMN apoptosis (A. Lee, personal communication; Lee, 1993); TNF-α (1000 units/ml) significantly increased PMN apoptosis (J. Murray, personal communication). IL-4 was active on monocyte cultures themselves, promoting shape change whilst TGF-β (1 ng/ml) promoted eosinophil apoptosis (L. Meagher, personal communication). Other agents investigated were: cycloheximide, ethanol, db-cAMP and dexamethasone.

2. 7. 3. 3 Reactive Oxygen Intermediates and Antioxidants.

For all these experiments cells were incubated at 1 x 10^6 cells/ml in 8 well covered slides at a final volume of 400 µl/well for PMN and 200 µl/well for Mø. Cells were incubated for 8 or 24 hours in standard conditions in the presence or absence of H_2O_2 as the ROI, with or without addition of antioxidants. As serum contains significant antioxidant capacity all

incubations were performed in the presence of 2% serum unless stated. H_2O_2 was purchased as a 30% stock solution and used at final concentrations between 0.1 and 5 mM, stock concentration at the time of use was confirmed by measuring absorbency at 230 nm. Antioxidants were used at a final concentration of 10 mM for Trolox and 2 mM for GSH. Trolox, a soluble α tocopherol (vitamin E) analogue, was prepared fresh for each experiment, it was dissolved in IDMEM by sonication and the pH adjusted to 7.4 with sodium bicarbonate.

2. 7. 3. 4 Antioxidant Capacity of Serum.

Serum from each donor was stored at -70°C for later measurement of the Trolox equivalent antioxidant capacity (TEAC), defined as the concentration of Trolox which had the equivalent antioxidant capacity to an equal volume of the solution under investigation. The normal range for human serum is 1.0 - 1.5 mM (Miller, 1993). Assay tubes were prepared with 497 µl PBS, 300 µl ABTS [2,2-Azino-bis (3-Ethylbenzthiazoline-6-sulfonic acid)], 36 µl metmyoglobin and 167 µl of H_2O_2 - giving final concentrations of ABTS of 150 µM, metmyoglobin of 2.5 µM and H_2O_2 of 75 µM, absorption at 734 nm of this mixture was determined after 12 minutes incubation. The presence of antioxidants quenches this absorption, hence a standard curve was constructed using 10 - 50 µl of Trolox (2.5 nMol/10 µl, adjusting the final volume of PBS to still yield 1000 µl) and the reduction of absorption of 10 µl of the test serum determined and related to that of Trolox.

2. 7. 4 Assessment of Apoptosis.

2. 7. 4. 1 DNA Electrophoresis.

The presence of DNA cleavage products was demonstrated using the Lee *et al.* modification of the methods previously described for lymphocytes (Trauth, 1989; Lee, 1993). Cells were washed and resuspended in 0.5 ml STE buffer (100 mM sodium chloride, 10 mM Tris, 0.1 mM EDTA pH 8.0) containing 1% sodium dodecyl sulfate (SDS) and 0.2 mg/ml proteinase K for 12 hours at 37°C. One DNA extraction with a phenol: chloroform: isoamyl alcohol mixture (25:24:1 v/v/v) and two with a chloroform: isoamyl alcohol mixture (24:1 v/v) were performed. Two volumes of ethanol and one tenth volume of 5 M sodium chloride were then added to precipitate the DNA which was stored at -20°C for 12 hours before being pelleted by centrifugation at 13 000 x g and resuspended in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). After 15 minutes predigestion with 100µg/ml ribonuclease at 37°C, samples were mixed with 0.1 volumes of loading buffer (TBE buffer containing 30% Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol). The DNA was electrophoresed in a 1.8% agarose gel in 0.5 µg/ml ethidium bromide for 30 minutes, along with a λDNA ECoR1/Hind III digest as size marker. When viewed under ultra violet light a ladder pattern indicated internucleosomal cleavage, resulting from endogenous endonuclease activation - the biochemical hallmark of apoptosis.

2. 7. 4. 2 Morphology.

2. 7. 4. 2a Light Microscopy.

PMN apoptosis was assessed by examining Dif Quik™ stained cytocentrifuge preparations under oil-immersion light microscopy at a magnification of x1000, apoptotic PMN being defined as cells containing

one or more darkly stained pyknotic nuclei (Savill, 1989a). Apoptotic monocytes and Mø proved to be much more fragile than PMN and cytocentrifugation destroyed their morphology, hence this method was not used for these cells (**Figure 3. 2**).

2. 7. 4. 2b Acridine Orange Fluorescent Microscopy.

Apoptosis in monocytes and Mø, PMN, lymphocytes and U937 cells was quantified by acridine orange fluorescence microscopy, another well recognized method for the detection of apoptosis (Arends, 1990; Stern, 1992). **Figure 3. 3** shows the characteristic morphology of apoptotic and non-apoptotic monocytes and Mø stained with acridine orange and viewed under green fluorescent light. Suspension cultured cells were resuspended, 10 µl placed on a microscope slide to which 5 µl of stock acridine orange solution (10 µg/ml) was added and protected with a coverslip. Cells were scored as apoptotic or non-apoptotic on the basis of their nuclear morphology as seen under green fluorescence and the percentage of apoptotic cells determined by counting >500 cells. For adherence cultures in 8 well slides, 1.5 µl of stock acridine orange solution (1 mg/ml) was added per well, incubated for 10 minutes and then viewed on an inverted fluorescent microscope at x 400. One thousand cells were counted, scoring as above. The number of cells per high power field was also recorded, counting ten or more fields per treatment. Fields were chosen at random with the field sufficiently out of focus to be unable to identify cell morphology, but to be able to identify the well boundaries.

2. 7. 4. 2c Electron Microscopy.

For examination by transmission electron microscopy a million cells were washed, pelleted, fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer, then treated with 1% osmium tetroxide and processed for transmission electron microscopy using standard techniques in collaboration with the electron microscopy facility, Department of Pathology, University of Edinburgh. Apoptotic cells demonstrated dense chromatin aggregation and cytoplasmic vacuolation whilst their plasma membrane remained intact (**Figure 3. 9**).

2. 7. 4. 3 Flow Cytometry.

2. 7. 4. 3a Quantification of Macrophage and U937 Cell Apoptosis.

The percent apoptotic Mø and U937 cells could be determined simply on the basis of forward and side scatter parameters using flow cytometry as verified in sections 3. 2. 8 and 4. 2. 1 respectively.

2. 7. 4. 3b Cytokine Receptor Expression.

Flow cytometry was used to distinguish non-apoptotic and apoptotic Mø and U937 cells on the basis of forward and side scatter, allowing the relative changes in surface molecule expression of these populations to be determined. Binding of antibodies from the cytokine receptor panel (section 5. 2. 1) was determined for apoptotic and non-apoptotic Mø that had been exposed to cycloheximide. Comparison of non-apoptotic, cycloheximide treated Mø and control Mø also allowed the relative effects of protein synthesis inhibition and apoptosis to be distinguished. For U937 cells, culture in serum free medium for 12 hours was used to induce apoptosis and apoptotic and non-apoptotic cells specifically gated on,

allowing relative cytokine receptor expression determined (**Figure 4. 1**). In all cases binding was related to that of a non-binding control antibody, MOPC-21C and to two positive controls; anti CD14 and HLA-DR (see section 3. 2. 9 and **Figure 5. 1**).

2. 7. 4. 4 Cell Counts.

To control for the possibility of phagocytosis of apoptotic Mø by non-apoptotic Mø, reducing the number of apoptotic cells observed, the final number of cells remaining in culture at the end of each experiment was determined, giving an indication of any reduction in cell number for each treatment. Suspension cultured cells were counted using a haemocytometer, following vigorous resuspension of the cells (>400 cells were counted per treatment and trypan blue exclusion was determined simultaneously). A correction was made for any loss of volume over the period of culture (always less than 10%) to correctly relate the initial and final counts. The Teflon foils were stained with Dif Quik™ after aspiration of the resuspended cells to identify whether any cells remained adherent. The number of adherent cultured cells (and nuclei) was estimated for each treatment well by counting the number of cells (and nuclei) per microscope field, a minimum of 8 fields were examined per treatment and the mean ± SE of the cells (and nuclei) determined. To control for the effect of giant cell formation by cell fusion, numbers were expressed as the number of nuclei per microscope field rather than the number of cells. By racking down from the surface, the number of non-adherent cells floating in each treatment well could be counted, very few of such cells were seen in any wells. Initially after counting under fluorescence microscopy, medium was aspirated, wells removed from the base of the slide which was fixed in methanol and Dif Quik™ stained. A

further count of the number of fixed stained cells per microscope field was undertaken, as a double check. This corresponded very closely with the acridine orange counts.

2. 8 Protein Synthesis Inhibition Assay.

This was performed according to the methods of Walt *et al.* (Walt, 1985). Briefly Mø were washed twice in methionine free MEM (Gibco), divided into three Teflon foils, each with 4 x 10^6 cells, suspended in MEM with 10% dialyzed FCS (to remove molecules of mw < 10 000). To each of these foils 3.14 ml ^{35}S (20 mCi) was added. In addition, foil a) had 18.7 µl of MEM added (control) foil b) had 1.87 µl of cycloheximide stock and 16.83 µl MEM (5 µM cycloheximide) and foil c) had 18.7 µl of cycloheximide stock added (50 µM cycloheximide). After 18 hours incubation, cells were harvested, counted and the percent apoptosis determined morphologically. Cells were washed three times, the supernatant saved each time and the final cell pellet was suspended in 200 ml PBS to which 15 ml 20% PBS was added. For protein precipitation this was heated to 95°C for 5 minutes, cooled, trichloroacetic acid added to a final concentration of 10% (24 ml of 100% trichloroacetic acid) and centrifuged at 13000 x g for 10 minutes at 4°C. The pellet was washed twice with cold ethanol and resuspended in 400 ml PBS, this pellet and all the washes were counted in a β counter.

2. 9 *In Vivo* Methods.

2. 9. 1 Mice and Inflammatory Challenge.

Mice BALB/c (H-2^d), C3HF/KAM (H-2^k) and their F1 hybrid, CB1 (H-$2^{k/d}$) breeding colonies were established locally, with the kind help of Mr. J. Verth, Department of Medical Microbiology, University of Edinburgh.

Female CB1 mice were used as donor mice and female C3HF/KAM as recipients for adoptive cell transfer experiments unless indicated, all mice were used between 11 and 15 weeks of age. Apart from one day of poor feeding and ruffled fur after inflammatory challenge, mice remained well throughout; of the mice used in the entire course of the experiments only one mouse died.

2. 9. 2 Inflammatory Challenge and Macrophage Kinetics.

Murine cell transfer experiments were performed with the help of Miss Helen Caldwell, Respiratory Medicine Unit, University of Edinburgh. The kinetics of the inflammatory cell response to the intraperitoneal (i.p.) challenge of two different sterile inflammatory agents, 2 ml of 1% starch or 2 ml of 4% thioglycollate (TG) (Difco, East Molesey, Surrey) was followed for 21 days (days 3, 5 and 10 for starch) to identify the resolution phase of inflammation in both CB1 and C3H/KAM mice. A minimum of 4 or more mice were terminally anaesthetized with ether at each time point for each strain and total cell number, viability and differential counts performed on peritoneal lavages.

2. 9. 3 Adoptive Cell Transfer.

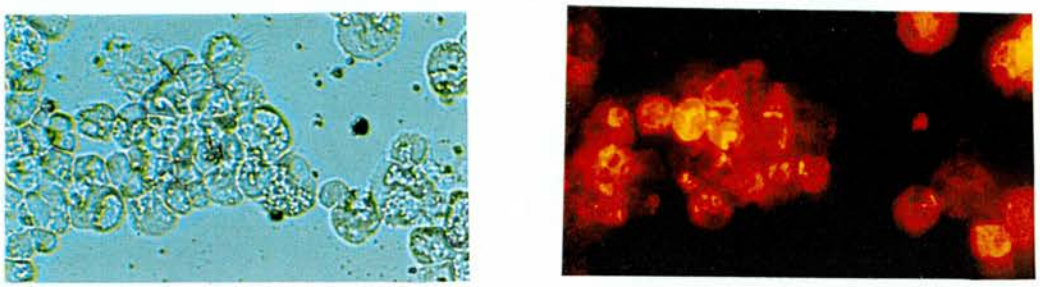
2. 9. 3. 1 Fluorescent Labelling of Cells.

Fluorescent labelling using the PKH26 cell linker kit (Sigma) allows stable cell labelling for prolonged periods without elution of dye or apparent alteration of cell physiology (Melnicoff, 1988a and b; Horan, 1990). Cells could be labelled *in vitro* using the red fluorescent cell linker dye PKH26-GL for general cell labelling or *in vivo* with the red fluorescent cell linker dye PKH26-PCL for phagocytic cell labelling. The fluorescent label was the same in both cases but the diluent differed.

Figure 2. 5 PKH26 Fluorescence after *In Vivo* and *In Vitro* Cell Labelling.

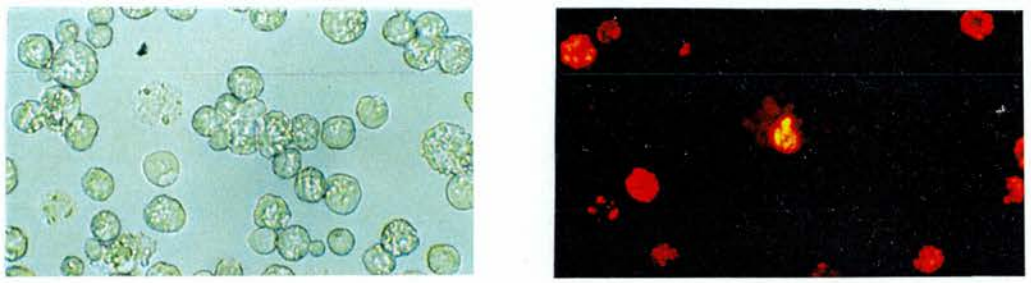
***In vitro* General Cell Labelling**

a b



***In vivo* Phagocytic Cell Labelling**

c d



Legend for Figure 2. 5 Photomicrographs of Mø after (**a** and **b**) *in vitro* general cell and (**c** and **d**)*in vivo* phagocytic cell labelling. This confirms that all cells are red fluorescent after *in vitro* general cell labelling as all cells that can be seen under light microscopy (**a**) are also seen under red fluorescent microscopy (**b**). As only a fraction of cells have incorporated the dye with *in vivo* phagocytic labelling, many cells seen under light microscopy (**c**) cannot be visualised under red fluorescent microscopy (**d**). The fluorescent label is the same for both labelling methods, only the diluent differs. With *in vitro* cell labelling the aliphatic fluorescent PKH26-GL chromophore is incorporated into the lipids of all cell membranes, whilst for phagocytic *in vivo* cell labelling, the diluent creates PKH26-PCL dye aggregates that need to be ingested and the degree of labelling depends on the volume of dye injected (section 7. 2. 2. 2).

The PKH26 dye is an aliphatic fluorescent chromophore that allows labelling in the lipids of the cell membrane for the general cell labelling whilst for phagocytic cell labelling, dye aggregates are formed and phagocytosed (Horan, 1990), thus all cells are labelled by the *in vitro* technique whilst only phagocytes are labelled using *in vivo* labelling (see **Figure 2. 5)**.

2. 9. 3. 1a ***In Vitro*** **Labelling.**

Free peritoneal cells were lavaged using 5 mls of sterile PBS and counted, then aliquots containing 1 x 10^7 cells were transferred into 15 ml polypropylene tubes and washed. Meanwhile 3 μM dye (2x stock dye) was prepared by diluting stock with Diluent C (supplied by the manufacturer as part of the kit). The cell pellet was resuspended in 1 ml of Diluent C, which was rapidly added to 1 ml of 2x stock dye, incubated for 2 minutes and the labelling stopped by addition 2 mls of FCS. Cells were washed three times, transferring to a fresh tube each time.

2. 9. 3. 1b ***In Vivo*** **Labelling.**

Under sterile conditions 100 μl of dye was mixed with 900 μl of absolute ethanol, 50 μl of this was dissolved in 10 mls of Diluent B (provided by the manufacturer as part of the kit). Using a fresh sterile syringe and 26 gauge needle each time, 0.5 - 2 mls was injected into the peritoneal cavity of donor mice 1 - 24 hours prior to sacrifice, control mice received an equal volume of PBS. Since PKH26-PCL had not previously been used for i.p. labelling during inflammation, there was no information from the manufacturers on labelling protocols, hence I tried 1, 1.5, 2, 18 and 24 hours labelling prior to lavage and injection of between 0.5 and 2 ml i.p. PKH26-PCL. No difference in the labelling intensity, percentage cells

labelled, cell recovery or viability was apparent between these labelling times and 1.5-2 hours was chosen for future experiments. Increasing the injectate volume increased the percent cells labelled but with 2 mls PKH26-PCL the cell viability decreased as discussed in section 7. 2. 2. 2.

2. 9. 3. 1c Analysis of PKH-26 Fluorescent Cells.

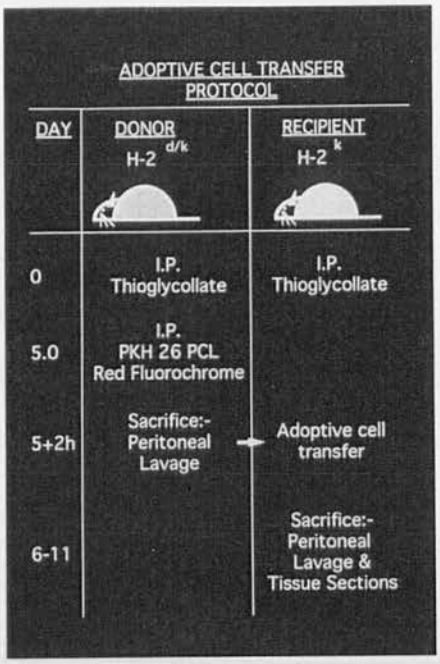
Flow cytometric analysis was used to analyze *in vitro* and *in vivo* PKH-26 cell labelling efficiency. This confirmed that the *in vitro* method labelled all cells, Mø, lymphocytes and granulocytes, equally, whilst the *in vivo* method labelled only phagocytic cells as expected (**Figure 2. 5**). As by the 5th day post inflammation there were less than 3% PMN, *in vivo* labelling was essentially restricted to Mø. This analysis also showed that upon i.p. injection of 0.5 mls PKH-26 PCL, only approximately 1/3 of the cells took up the dye, as discussed in section 7. 2. 2. 2 but that the percent Mø labelled increased to 74% using 1 ml dye. In certain experiments spleen and selected lymph nodes were collected in PBS, washed vigorously 4 times and disrupted either mechanically or by enzymatic digestion with 0.5 mg/ml collagenase for 15 minutes. The resultant cell suspension was analyzed by flow cytometry for the presence of PKH26 red fluorescence.

2. 9. 3. 2 Protocol for Adoptive Cell Transfer.

This protocol was designed with help from Dr. Sarah Howie, Department of Pathology, University of Edinburgh. Peritoneal inflammation was induced by i.p. TG or starch in both donor and recipient mice 5 days prior to cell transfer, such that both groups of mice were at the same stage of inflammation. For adoptive transfer of *in vitro* labelled Mø, the peritoneal cavities of donor mice were lavaged 5 days after inflammatory

challenge, recovered cells were labelled, counted and viability determined, then between 5 and 30 x 10^6 cells resuspended in 2 mls PBS were injected into the peritoneal cavity of recipient mice using a 21 gauge needle. For adoptive cell transfer after *in vivo* labelling, donor mice underwent peritoneal lavage 1.5 hours after PKH26-PCL injection. Lavaged cells were suspended in PBS with 10% FCS at 4°C, washed twice (250 x g), counted and viability determined. In certain experiments cells were fixed in 2% formalin for 5 minutes and washed a further three times prior to transfer. Cells (live or fixed) in 2 ml PBS at the concentrations stated were re-injected into the peritoneal cavity of recipient mice (at the same stage of inflammation as the donors) using a 21 gauge needle. In all cases the donor cells were >85% Mø, the remaining cells were mainly lymphocytes, the viabilities differed between labelling procedures however. With *in vivo* labelling, viabilities were always >95% - whilst considerable problems were encountered in *in vitro* labelling. Initial poor viabilities during *in vitro* labelling may have been due to excessive spin speeds during washing (using the gravitational force as recommended by the manufactures - 400 x g), however with slower speeds viabilities of <80% were still encountered. These were improved by reducing the concentration of the stock dye, however the best viabilities were still only 90%. For this reason *in vivo* labelling was chosen for the rest of this work. After cell transfer, recipient mice were sacrificed at various times between 4 hours and 1 week later, their peritoneal cavities lavaged and tissue taken for histology and fluorescence studies (summarized in **Figure 2. 6**). Some mice received unlabelled inflammatory Mø to serve as controls for flow cytometry and frozen section studies.

Figure 2. 6 Protocol for Adoptive Cell Transfer.



MACROPHAGE LABELING PATTERNS.	RED +	GREEN (FITC) +	
	PKH 26 PCL Fluorochrome	Anti d	Anti k
DONOR (H-2 d/k) PKH d k	40%	Yes	Yes
HOST (H-2 k) k	No	No	Yes
DONOR PHAGOCYTOSED BY HOST PKH k	40%	No	Yes

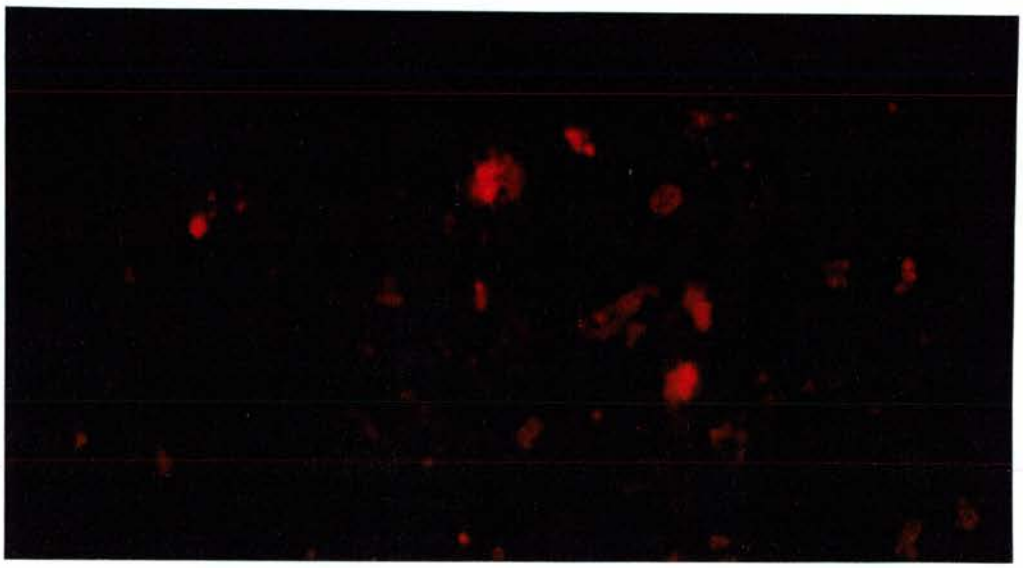
Legend for Figure 2. 6 Summary of the experimental protocol employed. Both donor (H-$2^{d/k}$) and recipient (H-2^{k}) mice received sterile inflammatory challenge at the same time. Five days later donor mice received i.p. injection of PKH26-PCL to label phagocytic cells *in vivo*. These donor cells (>85% Mø, 27 - 42% PKH26-PCL labelled) were lavaged and injected into the peritoneal cavity of recipient mice at the same stage of resolving inflammation as the donor mice. These mice were sacrificed between 4 and 168 hours later, their peritoneal cavities lavaged and tissue taken to determine the distribution of donor cells. The possible labelling patterns of Mø in peritoneal lavage of recipient mice are shown.

2. 9. 4 Tissue and Cell Staining.

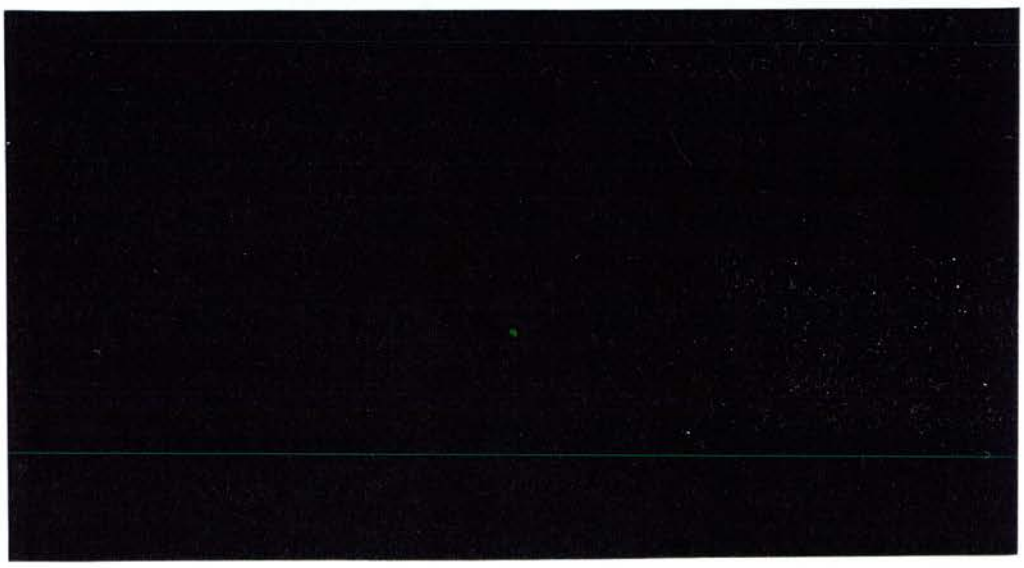
Tissue for frozen section was immediately snap frozen in liquid nitrogen, 3 μM sections were cut, mounted on slides and the distribution of the PKH26 label determined under red fluorescence using an Olympus BH-2 RFCA fluorescent microscope. Specificity of labelling could be confirmed by viewing the section under green fluorescent light as PHK26 labelled cells were only red fluorescent (**Figure 2. 7**), occasional artifact was both red and green fluorescent. Staining for the presence of H-2^d was also performed on these sections, however as fixation abolished PKH26 fluorescence, an unfixed section was viewed first for the presence of PKH26 before the remainder were fixed. Despite attempts using a range of fixatives (alcohol, acetone, paraformaldehyde and formalin), no useful double labelled sections were obtained due to loss of dye. Sections were stained in a Sequenza™ (Shandon, Pittsburg PA) to reduce background staining. Slides were blocked sequentially with NMS, Avidin D and biotin blocking solution (Vector Laboratories, Bretton, Peterborough) before addition of biotin conjugated mouse anti-mouse H-$2D^d$ mAb (Pharmingen). Slides were washed and ABComplex/AP (avidin/ biotinylated alkaline phosphatase kit, Dako) and Vector® Red alkaline phosphatase substrate kit added (Vector Laboratories) before counterstaining with haematoxylin. Control slides were stained in the absence of the biotinylated anti-H-2^d mAb; tissue from H-$2^{k/d}$ mice acted as positive controls and from H-2^k mice as negative controls in each run. Formalin-fixed tissue was paraffin embedded, cut sections were stained with haematoxylin and eosin.

Figure 2. 7 Specificity of PKH26-PCL Red Fluorochrome Labelling.

i) Red Fluorescence.



ii) Green Fluorescence.



Legend for Figure 2. 7 Tissue section of parathymic LN 48 hours after i.p. adoptive cell transfer of 15 x 10^6 semi-allogeneic cells (>85% Mø and 40% PKH26-PCL labelled) showing clear definition of cells in the LN viewed under red fluorescent light but no cross-over when viewed under green fluorescent light. Any occasional artefact could be distinguished as this would be visualised under both red and green fluorescence (x 200).

2. 9. 5 Cell Culture for *In Vivo* Studies.

2. 9. 5. 1 BHK Plates.

To obtain the very pure populations of inflammatory Mø needed to confirm gating parameters and immunofluorescence, an adherence method was employed to enrich 5 day peritoneal lavage cells for inflammatory Mø. Coating of tissue culture plastic with matrix proteins can alter the adherent substrate; this was used to improve Mø detachment (Ackerman, 1978). BHK cells (BHK 21, clone 13: ECACC) were grown to confluence on 140 mM tissue culture dishes in Glasgow's BHK-21 medium containing 10% Tryptose phosphate broth (Gibco) and 10% FCS. Treatment of the monolayer with 10 mM EDTA easily detached cells in sheets, after three washes the sterile matrix coated plates were stored at -70°C until used. Peritoneal lavage cells were cultured on pre-warmed BHK matrix coated plates in IDMEM at 2 x 10^6 /ml for 1 hour. Non-adherent cells were washed off and the adherent cells then lifted off by 5 minutes incubation with 5 mM EDTA on ice (**Figure 2. 4b**). These cells were >94% Mø by non-specific esterase staining and indirect immunofluorescence. Low recovery and possible activation following adherence precluded their use in adoptive cell transfer experiments.

2. 9. 5. 2 *In Vitro* Culture of PKH-26 Labelled Peritoneal Macrophages.

To determine the retention of PKH26 label and the effect of PKH26 labelling on cell viability with time, fresh and formalin fixed cells were cultured in IDMEM supplemented with 100 units penicillin, streptomycin and 10% FCS, in hydrophobic Teflon foils at 37°C in 5% CO_2. Samples were removed daily for viability, PKH26 (GL or PCL), H-$2^{k/d}$ expression and differential cell counts. The effect of brief formalin

fixation on donor cell function was confirmed by examining cell adherence to tissue culture treated plastic slides, live cells were strongly adherent whilst fixed cells were completely non-adherent.

2. 10. Statistical Analysis.

Statistical analysis was performed in collaboration with Dr. W Adams, Medical Statistics Unit, University of Edinburgh. The percentages of PKH26 labelled cells , H-$2^{k/d}$ cells and phagocytosed cells in peritoneal lavage were logarithmically transformed using the equation

$$y = \log_e (1 + 10p)$$

where p = the percentage. This converted the values into a normal distribution allowing calculation of geometric means and 95% confidence intervals. The log means and variance are shown below for the transfer of live and formalin fixed PKH26-PCL labelled cells.

	Log Mean (m) for each time point					**Variance**
	24	**48**	**72**	**96**	**168 (hours)**	**(s)**
Live cell transfer:						
% PKH26-PCL	4.71	4.2	4.02	2.49	3.21	0.533
% H-$2^{k/d}$	5.7	5.07	5.02	0	1.48	0.646
% Phagocytosed cells	2.24	2.12	1.42	0.64	3.21	0.505
No. Mice	8	4	5	5	7	
Fixed cell transfer:						
% PKH26-PCL	4.33	-	3.69	4.52	2.85	1.92
% H-$2^{k/d}$	2.41	-	0.54	1.85	0.87	2.08
% Phagocytosed cells	4.31	-	3.55	3.89	3.29	1.81
No. Mice	6	-	6	6	5	

The plots of percentages shown (**Figures 7. 5, 7. 6** and **7. 7**) were achieved by transformed data back into linear scale, the conversion being:

$$p = \frac{e^{y} - 1}{10}$$

These plots suggested that there was a linear decline was to zero by 96 hours for labelled donor cells after live cell transfer (see section 7. 2. 3) but not for fixed cells (see section 7. 2. 4). With 4 equi-spaced time points (24, 48, 72 and 96 hours), linearity can be tested for by means of orthogonal (independent) polynomials obtained via ANOVA. For the 4 points there are 3 components, linear, quadratic and cubic; if points lie on a straight line then the linear component will be significant and the others will not. Using the following equation:

$$t_v = \frac{l_1m_1 + l_2m_2 + l_3m_3 + l_4m_4}{\sqrt{s^2 (l^2_1/n_1 + l^2_2/n_2 + l^2_3/n_3 + l^2_4/n_4)}}$$

t tests for each time can be determined, where v is the time after cell transfer, l is a weighting for each of the components as shown in the table below, m are the log means for each time point, n the number of mice at each time point and s the variance.

	Linear	**Quadratic**	**Cubic**
l_1	-3	1	-1
l_2	-1	-1	3
l_3	1	-1	-3
l_4	3	1	1

For example, after transfer of live PKH26-PCL labelled Mø the linear component of the equation reads:

$$t_{24} = \frac{-3 \times 4.71 - 4.20 + 4.02 + 3 \times 2.49}{\sqrt{0.533 (9/8 + 1/4 + 1/5 + 9/7)}}$$

Using this we see that:

	PKH26 labelled Mø after live cell transfer (prob.)	**PKH26 labelled Mø after formalin fixed cell transfer (prob.)**
Linear	0.007	0.126
Quadratic	0.189	0.436
Cubic	0.334	0.219

Only the linear component is significant for live cell transfer, none are for fixed cell transfer. Hence the decline of PKH26-PCL labelled cells after live adoptive transfer was linear to 96 hours but no such trend existed for formalin fixed cells.

Regression analysis was employed to determine whether there was concordance of the curves at 96 hours after cell transfer of 15 or 30 million donor cells (section 7. 2. 3). The total number of Mø free in the peritoneal cavity at each time point for CB1 and C3H/KAM mice were compared using ANOVA. A difference in the recovery rates of labelled cells in the presence or absence of inflammation was tested for using Wilcoxon's rank sum test for non-parametric data. All tables and graphs display the mean ± the standard error (SE) of the mean unless otherwise stated. For comparison between treatments of *in vitro* cells, Students t test was used unless otherwise indicated.

Chapter 3

CHARACTERIZATION OF MONOCYTE AND MACROPHAGE APOPTOSIS.

3.1 INTRODUCTION.

A reproducible model to allow investigation of the controls on monocytes and Mø survival *in vitro* required the reliable isolation of monocytes from peripheral blood, the *in vitro* culture of Mø and the identification and quantification of monocyte and Mø apoptosis. Although several systems for monocyte purification have been described, the morphology, characteristics and quantification of Mø apoptosis had not been described.

The most common and accepted methods for determining and quantifying apoptosis are based on morphological criteria. Light microscopy of stained cytological preparations (Savill, 1989a; Dransfield, 1994), fluorescence microscopy using acridine orange or DAPI (McConkey, 1988; Gregory, 1991) and electron microscopy (Wyllie, 1984; Savill, 1989a) have all been used to identify and quantify apoptotic cells. Traditional measures that distinguish live from necrotic cells, such as exclusion of trypan blue are not sensitive for cells in the initial stages of apoptosis as they are still able to exclude vital dyes, a capacity which may be preserved late into the life of the apoptotic body (Haslett, 1994). Gel electrophoresis of DNA can be used to confirm endonuclease activity associated with apoptosis and it is possible to quantify this either by separating fragmented and intact DNA (Wyllie, 1980; Sellins, 1987) or by using densitometry techniques (Waring, 1990). Apoptotic cells can also be distinguished by flow cytometry using propidium iodide or other DNA binding fluorochromes (Ojeda, 1990; Nicoletti 1991; Telford, 1994) and by the technique of terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end-labelling or TUNEL, facilitating immunohistochemical

staining of apoptotic cells at the single cell level in tissue sections (Gavrieli, 1992; Tornusciolo, 1995).

Mangan *et al.* provided a clear picture of monocyte apoptosis, documenting DNA fragmentation and demonstrating the correlation with loss of cell volume, propidium iodide uptake and characteristic electron microscopy findings (Mangan, 1991a and b). Although Mø apoptosis had been described in response to ATP treatment (Hogquist, 1991a), co-culture with cytotoxic T lymphocytes (Zychlinsky, 1991) and protein synthesis inhibition (Waring, 1990), this had only been quantified by using DNA fragmentation. My preliminary work showed that morphological assessment of monocyte and Mø apoptosis was difficult; apoptotic monocytes and Mø proved too fragile to withstand cytocentrifuge preparation (**Figure 3. 2**) and flow cytometry using PI was found to be not generally applicable as some Mø populations, especially alveolar Mø, had high autofluorescence that obscured any selective uptake of PI.

It has been clearly established that Mø specifically recognize and ingest apoptotic cells, a novel recognition mechanism for this has been described (Savill, 1989a and b, 1990 and 1992; Fadok, 1992a). This suggests that cell surface changes during the process of apoptosis allow for recognition and removal of apoptotic cells by Mø, but not by monocytes. As a description of the morphology of Mø apoptosis was lacking, the surface molecular changes associated with monocyte/Mø apoptosis had also not been investigated.

My aims initially were:

- To find the most suitable method for monocyte preparation and Mø culture.
- To establish a methodology for assessment of apoptosis in monocytes and Mø.
- To establish a system for the reproducible induction of apoptosis allowing characterization of the apoptotic process in Mø with particular reference to their cell surface characteristics.
- To investigate the possibility that Mø phagocytose apoptotic Mø and monocytes within the same culture.

3. 2 RESULTS.

3. 2. 1 Purity of Monocyte and Macrophage Populations.

3. 2. 1. 1 Monocytes.

Monocytes isolated by CCE or by discontinuous Percoll gradient centrifugation and plasma plate adherence were of similar purity and initial viability 91.2% (range 80 - 96%; >99% viable, n = 31) for CCE compared with 90.2 % (range 84.6 - 96%; >99% viable, n = 81) for density gradient centrifugation. Average yields from CCE were 30.2 x 10^6 cells/preparation (range 15.6 - 44 x 10^6) when 216 mls of blood were taken from each donor, thus providing 1.40 x 10^5 monocytes per ml of blood. Density gradient centrifugation and adherence provided a mean of 21.5 x 10^6 cells/preparation (range 10.5 - 30 x 10^6), again 216 mls of blood were taken, each donation yielding 1.00 x 10^5 monocytes per ml of blood. The mean mononuclear count after dextran sedimentation and density gradient centrifugation of 216 mls blood was 165 x 10^6 cells of which 20.7% (range 12.4 - 29.9%) were monocytes as determined by flow cytometry.

Thus using plasma plate adherence 62.5% of the total monocytes were recovered, CCE provided a better monocyte recovery at 88% of the total.

3. 2. 1. 2 Macrophages.

Monocytes cultured in IDMEM with 10% autologous serum in suspension culture or adherent to 24 well plates matured into Mø over 5 days. The purity of these cultures were as follows:

92.9% (range 85.6 - 98%, n = 73) assessed by Dif Quick™ stained cytospins;

93% (range 91 - 96%, n = 5) assessed by non-specific esterase staining;

92.5% (range 89-98%, n = 48) assessed by flow cytometry using forward and side scatter parameters;

90.2% (range 84.3 - 93.8%, n = 24) assessed by flow cytometry using indirect immunofluorescence to determine CD11a/CD18, CD14 and HLA-DR expression.

Mø viability was greater than 99% determined by trypan blue exclusion. The non-adherent cells from 24 well plates were 96% Mø after 5 days and the remaining adherent cells, after lifting off with 5 mM EDTA, were 98% Mø as assessed both by cytospin and flow cytometry characteristics (n = 4).

3. 2. 1. 3 Adverse Effects of Elutriator on Monocyte Viability.

The initial viability of monocytes and corresponding Mø yields after 5 days in cultures were similar whether the cells were prepared by discontinuous Percoll gradient centrifugation or by CCE. However after 4 months of elutriator use and for reasons as yet unclear, the viability of monocytes isolated by CCE declined rapidly during *in vitro* culture, with less than 10% of cells still viable after 48 hours. Monocyte death occurred without any evidence of infection and was not prevented by rigorous cleaning and sterilizing of the elutriator, changing all the tubing and the

chamber, or by altering the percent autologous serum in the elutriation medium from 0 to 2%. In an attempt to eliminate any possible problems with elutriator hardware an alternative rotor was used without improvement in monocyte viability. Maintaining the elutriation medium on ice had only a slight beneficial effect. Elutriation medium that had passed through the elutriator did not reduce the viability of cells prepared by discontinuous gradient centrifugation. Greater than 80% of elutriated U937 cells were viable after 48 hours, however these cells were maintained in the elutriator for less than an hour while monocyte elutriation often lasted 80 - 90 minutes. PMN prepared by CCE had similar survival to those prepared by discontinuous gradient centrifugation and were in the elutriator for longer than monocytes. Because of this unexplained pro-apoptotic effect on elutriator prepared monocytes, cells were prepared by discontinuous Percoll gradient centrifugation and plasma plate adherence for the remainder of the project.

3. 2. 2 Changing Concentration of Monocytes in Culture Affects Macrophage Yield.

To determine the optimum concentration at which to culture monocytes in suspension for maximal five day Mø yield, eight experiments were performed. Monocytes were cultured at initial concentrations of 0.1, 0.5, 1.0, 2.0 and 5.0 x 10^6/ml, the results for each experiment are shown in **Table 3. 1**. Maximum Mø yields occurred when monocytes were seeded at an initial concentration of 1 x 10^6/ml, hence this was the concentration used throughout the rest of this project. In **Table 3. 1** we see that comparison of Mø recovery starting with 0.1 x 10^6 monocytes/ml with that from 0.5 to 5.0 x 10^6/ml, using Students paired t test, shows that only

an initial monocyte concentration of 1.0 x 10^6/ml results in a significantly greater Mø recovery. Recovery from starting concentrations of 0.5 to 5.0 x 10^6/ml do not differ significantly from each other.

Table 3. 1

Effect of Altering the Initial Culture Concentration of Monocytes on Final Yield of Macrophages.

Initial Concentration of Monocytes (10^6/ml)	% Macrophages Recovered {experiments a - h}. a) b) c) d) e) f) g) h)	Mean ± SE	p
0.1 x 10^6/ml	32, 22, 25, 21, 36	27.2 ± 2.9%	-
0.5 x 10^6/ml	40, 31, 22, 34, 52	35.8 ± 5.0%	n/s
1.0 x 10^6/ml	48, 39, 34, 67, 45, 53, 21, 41	43.5 ± 4.5%	<0.05
2.0 x 10^6/ml	51, 37, 33, 61, 23, 40, 27, 29	37.6 ± 4.5%	n/s
5.0 x 10^6/ml	51, 33, 31, 51, 28, 40, 22, 39	36.9 ± 3.7%	n/s

Legend for Table 3.1

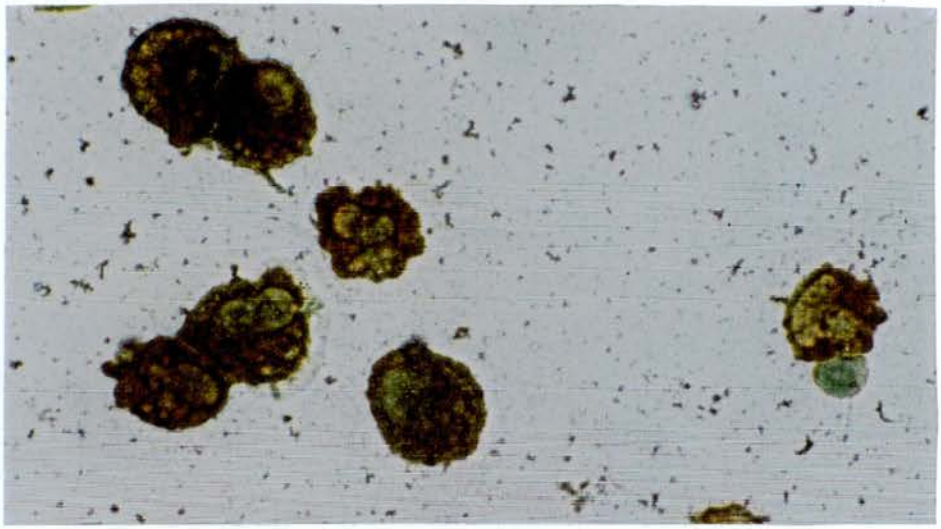
Results of varying the initial concentration of monocytes on the final yield of Mø after 5 days culture. Mø yield as a percent of the initial monocyte concentration are shown for 8 separate experiments (only 5 for concentrations 0.1 to 5.0 x 10^6 /ml), labelled a) to h), mean ± SE for each initial monocyte concentration shown on the right.

3. 2. 3 Assessment of Constitutive Monocyte/Macrophage Apoptosis.

Culturing human peripheral blood monocytes for 5 days yielded functionally mature Mø with typical expression of non-specific esterase

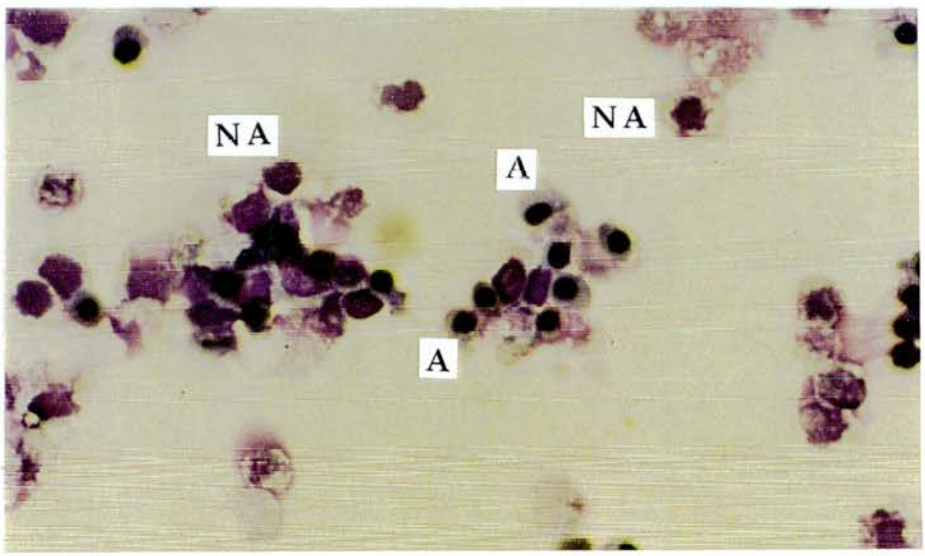
(**Figure 3. 1**) and loss of peroxidase activity (section 6. 2. 5), surface adhesion molecule expression (section 3. 2. 9) and ability to phagocytose opsonized zymosan and apoptotic PMN after 3 days in culture (section 3. 2. 10). There was a steady attrition in monocyte numbers over this culture period with recovery of approximately 46.5% (n = 24) of the original number cultured. The decline in cell number was relatively constant each day over this five day period. Unlike apoptotic PMN, apoptotic Mø did not retain their morphology during cytospinning, as shown in **Figure 3. 2**, thus this could not be used to quantify apoptosis in these cells. Propidium iodide (PI) staining, a commonly used technique to identify and quantify apoptotic cells (Nicoletti, 1991) was of limited use for Mø as the well known autofluorescence of Mø increased steadily during differentiation and was especially high in alveolar Mø (Viksman, 1994), effectively obscuring the degree of uptake of PI. Staining these cells with acridine orange (Arends, 1990) however proved to be a very sensitive method to demonstrate those cells with apoptotic morphology (**Figure 3. 3**) and allowed quantification of apoptosis. The percent of Mø exhibiting apoptotic morphology at any one time was on average 4.3%, this remained relatively constant over the culture period as shown in **Table 3. 2**, and discussed in Appendix 1. Morphological counts assessed both apoptosis and necrosis, although necrotic cells were rarely seen and accounted for less than 0.05% of cells. Only a small decline in cell number could be accounted for by giant cell formation (i.e. cell fusion), in suspension culture this was relatively uncommon and only seen in the last 24 hours of culture when less than 5% of nuclei fused into giant cells.

Figure 3. 1 Non-specific Esterase Staining of Human Macrophages.



Legend for Figure 3. 1 Cytospin of human Mø stained for non-specific esterase as described in section 2. 7. 1, showing positively stained Mø (brown) and a small non-specific esterase negative lymphocyte (x 400).

Figure 3. 2 Fragility of Apoptotic Macrophages During Cytospinning.



Legend for Figure 3. 2 Cytospin preparation of cycloheximide treated Mø stained with Diff Quik showing many fragmented cells without nuclei. Many non-apoptotic (NA) and apoptotic (A) cells are seen but the large number of cells without clear morphology makes this an inappropriate method for assessment of Mø apoptosis (x 100).

Table 3. 2

Percentage of Apoptotic Monocytes in Suspension Culture.

Time (Hours)	Apoptotic Cells (%)	Total Cell Count (x 10^6)
0	2.1 ± 1.1	2.00
2	4.4 ± 0.8	ND
4	4.1 ± 0.5	ND
6	4.4 ± 0.9	1.90
8	4.2 ± 0.4	ND
10	4.0 ± 1.0	ND
12	4.6 ± 0.8	ND
18	4.5 ± 0.9	1.79
36	4.2 ± 0.7	1.64
72	3.9 ± 0.4	1.29
96	3.6 ± 0.9	1.15
120	3.7 ± 1.1	0.92

Legend for Table 3. 2

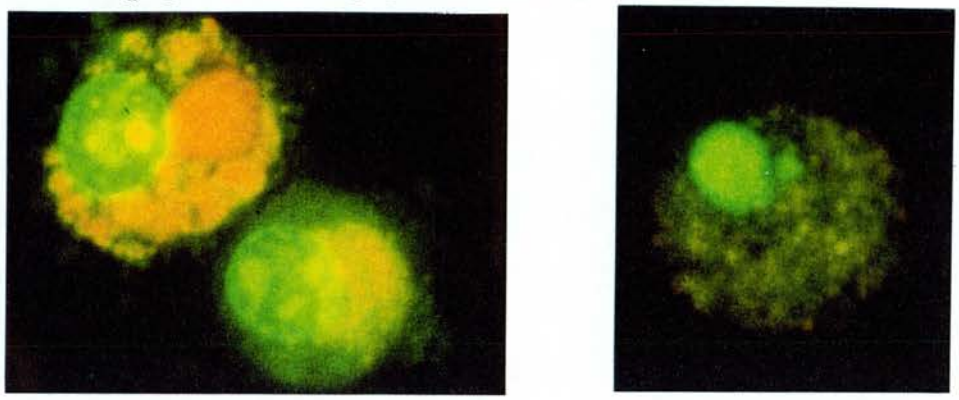
Monocytes were cultured in suspension in 10% autologous serum for five days and cell number and the percentage of apoptotic cells determined at the time points indicated. Apoptosis was determined by acridine orange staining and cell numbers by haemocytometer counts. Standard errors for cell numbers were all within 10% of the means; ND = not done. The percentage of apoptotic cells was significantly lower at the start than at any other time point ($p<0.05$), there was no significant difference between the percentage of apoptotic cells at the other time points. Results are expressed as mean

± SE, n = 8 for 0, 6, 18, 24, 36 and 120 hours and n = 3 for 2, 4, 8, 10, 12, 72, and 96 hours.

When 8 well slides of monocytes cultured for up to 72 hours were viewed following acridine orange staining, indistinct bodies that did not contain any nuclear material could be seen. These bodies, which were the size and shape of cells, were difficult to quantify as they were often very faint, there were never more than 4% per well except in the presence of cycloheximide when up to 12% could occasionally be seen (**Figure 3. 4**). They were never seen when cells cultured in Teflon foils were resuspended, placed onto a microscope slide and stained with acridine orange. Likewise, attempts at resuspending adherent cultured monocytes destroyed these bodies, suggesting that they were fragile and disrupted during resuspension. Due to the interaction of acridine orange and trypan blue, the ability of these bodies to exclude vital dyes could not be determined. Cycloheximide treatment increased the number of apoptotic cells and led to an increase in these indistinct "ghost cell" bodies. Furthermore the nuclei of apoptotic cells were seen to condense into smaller and smaller fragments. These "ghost cell" bodies probably represented late apoptotic bodies that had fully extruded their nuclear material, this is considered more fully in the discussion. Few "ghost cell" bodies were seen in Mø cultures and then only in the presence of cycloheximide. Also in the presence of cycloheximide, a number of non-apoptotic Mø could be seen to contain apoptotic cell bodies. This phagocytosis could be demonstrated both in suspension cultures using acridine orange fluorescent microscopy and in adherent cultures stained with Dif Quik™ (**Figure 3. 5**).

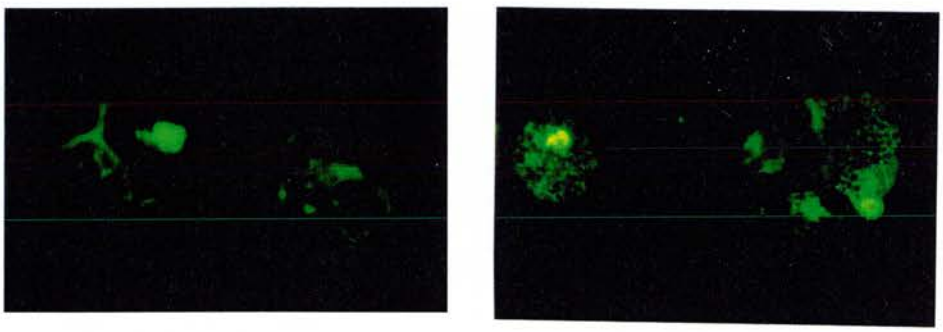
Figure 3. 3 Use of Acridine Orange Fluorescent Staining to Distinguish Apoptotic, Non-Apoptotic and Necrotic Macrophages.

i) Non-Apoptotic and Apoptotic Macrophages.



Legend for Figure 3. 3 i Suspension cultured human Mø were exposed to 50 μM cycloheximide for 18 hours to induce apoptosis in a proportion of cells. They were stained with acridine orange and viewed under green fluorescent light (section 2. 7. 4. 2). Two typical non-apoptotic Mø, with large open nuclei are shown on the left and the condensed characterless nucleus of an apoptotic Mø on the right (taken at x 400).

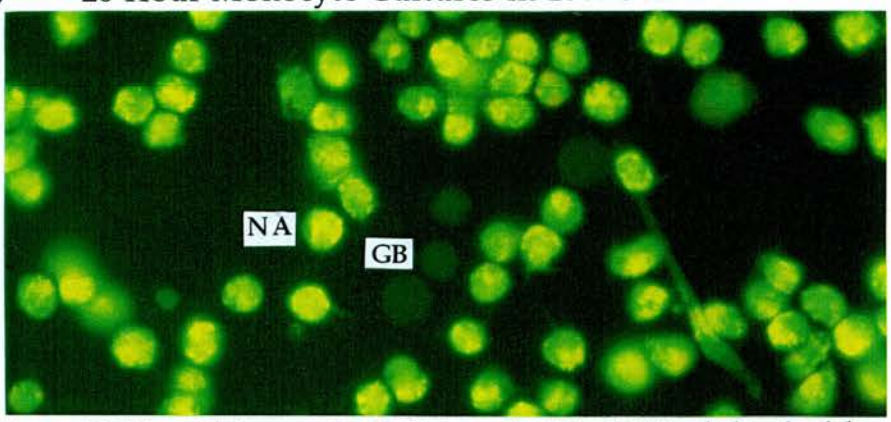
ii) Necrotic Macrophages.



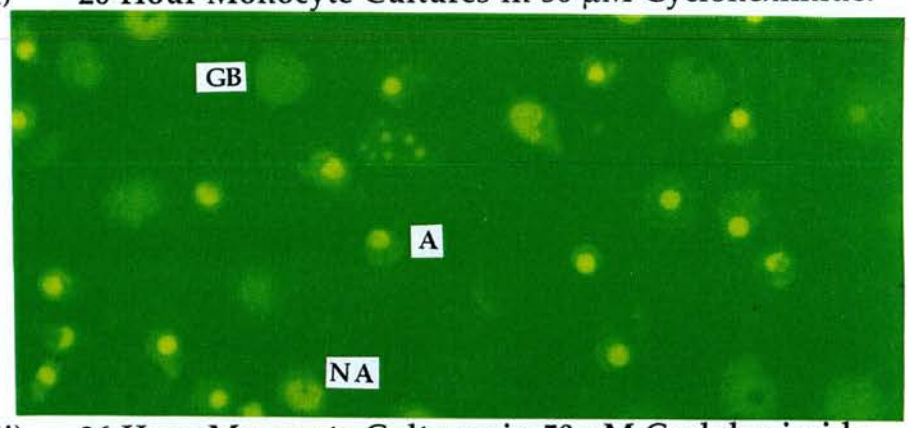
Legend for Figure 3. 3 ii Mø from the same donor were exposed to 2 cycles of freeze/thawing to induce necrosis and were again stained with acridine orange. Pictured are 4 examples demonstrating the clear morphological differences between necrosis and apoptosis. Necrotic cells are swollen, the internal architecture of the cell is disrupted and they have lost cytoplasmic membrane integrity (taken at x 200).

Figure 3. 4 Monocyte: Apoptosis and "Ghost Cell" Bodies.

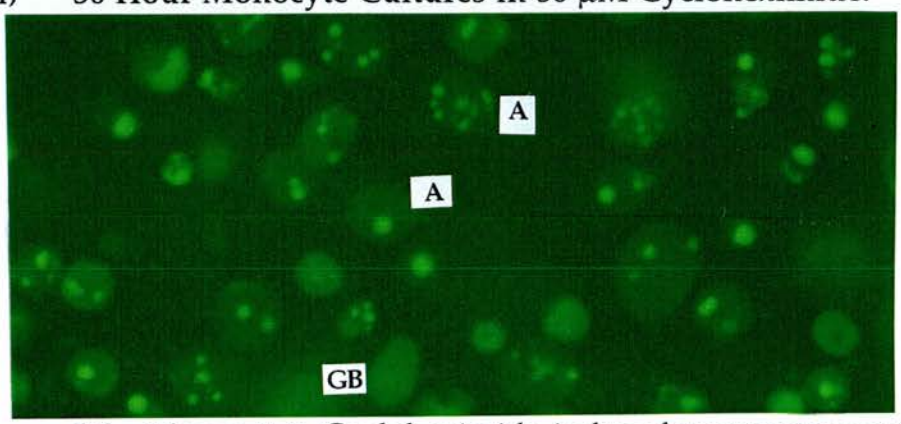
i) 26 Hour Monocyte Cultures in 10% Serum.



ii) 26 Hour Monocyte Cultures in 50 μM Cycloheximide.



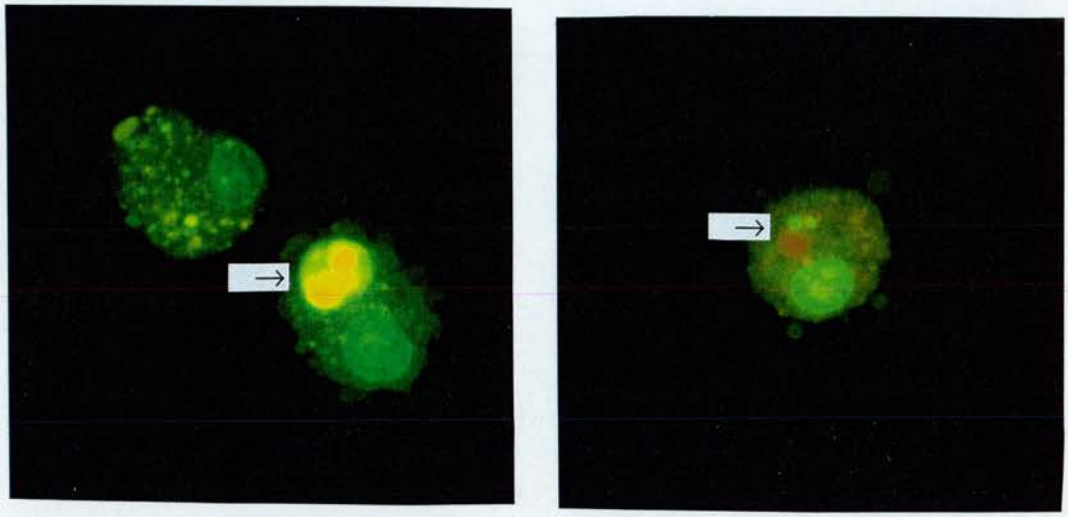
iii) 36 Hour Monocyte Cultures in 50 μM Cycloheximide.



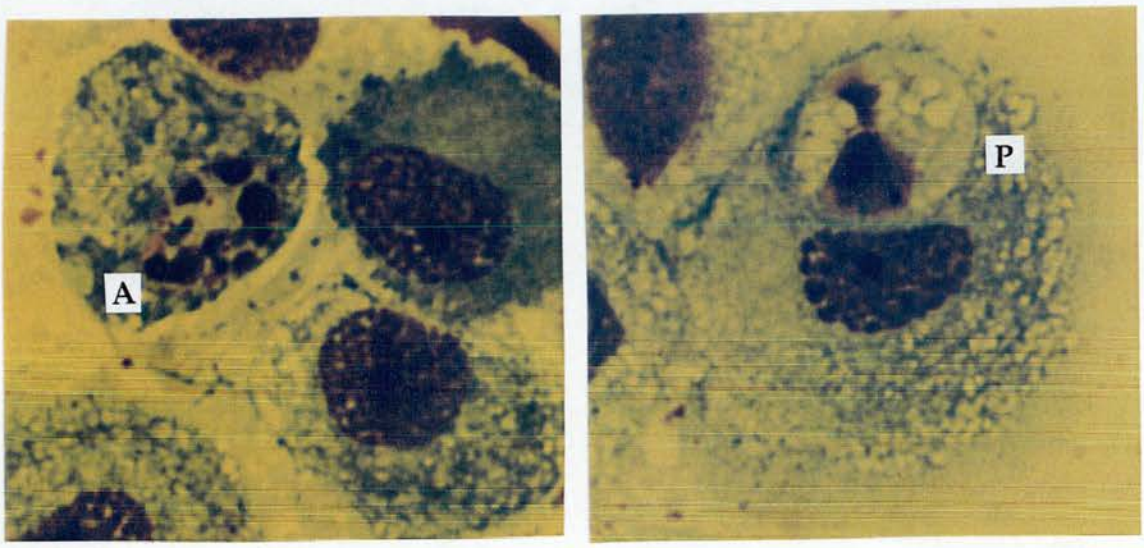
Legend for Figure 3. 4 Cycloheximide induced monocyte apoptosis; non-apoptotic (NA) cells have an open nucleus, apoptotic cells (A) have condensed characterless nucleus and "Ghost cell" bodies (GB) are indistinct bodies without nuclear material. Note the multiple fragmented nuclear bodies in the 36 hour cycloheximide culture - increasing nuclear disintegration is a feature of late apoptosis (x 200).

Figure 3. 5 Macrophage Phagocytosis of Apoptotic Macrophages.

i) Acridine Orange Fluorescent Microscopy of Macrophages in Suspension Culture Showing 2 Examples of Phagocytosed Apoptotic Cells (Arrows).



ii) Diff Quik Stained Adherent Cultured Macrophages Demonstrating Apoptotic Macrophage (A) and Phagocytosis of Apoptotic Cell (P).



(All taken at x 400)

3. 2. 4 Apoptosis vs. Necrosis: Comparison of Morphology.

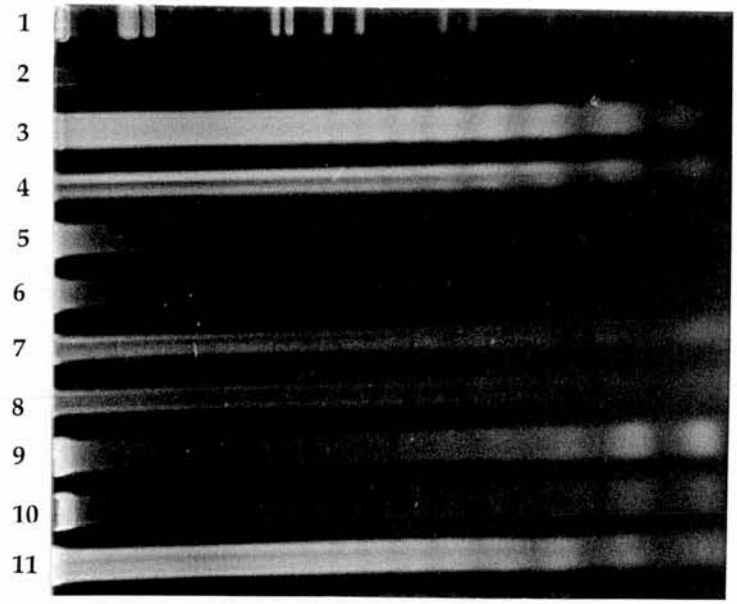
To ensure that the distinct morphological changes accompanying apoptosis could be distinguished from those of necrosis, a population of Mø were divided, half were exposed to 50 μM cycloheximide to induce apoptosis and the other half underwent two freeze/thaw cycles to induce necrosis. The differences between these cell populations were clear and unmistakable when viewed by acridine orange fluorescent microscopy and conformed to the morphological criteria previously used to identify these states (**Figure 3. 3**). Furthermore necrotic Mø remained clearly distinguishable when viewed 4 hours later indicating that they were relatively robust.

3. 2. 5 Characterization of Cycloheximide Induced Apoptosis In Human Macrophages.

3. 2. 5. 1 DNA Electrophoresis Data.

Mø, cultured either in suspension (n = 14) or adherent to 24 well plates (n = 4), were exposed to cycloheximide (50 μM), an equal volume (5 μl/ml) of ethanol (the diluent for cycloheximide) or medium for 18 hours. In all experiments gel electrophoresis demonstrated that only the addition of cycloheximide induced characteristic DNA fragmentation associated with apoptosis (**Figure 3. 6**). DNA fragmentation was time dependent in that no laddering could be seen after 5 minutes but was apparent after 2 hours (n = 3) of cycloheximide treatment and pronounced laddering was apparent after 8 hours (n = 4) and still detectable after 40 hours (n = 2). Cycloheximide also specifically induced DNA fragmentation in 8 and 9 day old cultures of murine bone marrow derived Mø (n = 6) and in alveolar Mø (n = 4).

Figure 3. 6 DNA Gel Electrophoresis Confirming Cycloheximide Induces Macrophage Apoptosis.



Legend for Figure 3. 6 Photomicrograph of ethidium bromide stained agarose gel viewed under ultraviolet light with DNA from 1 x 10^6 cells/lane, extracted as described in section 2. 7. 4. 1. Ladder pattern typical of apoptosis seen in lanes 3, 4 and 7 - 11. Lanes are as follows:

1 λ DNA ECoR1/Hind III digest - size marker.

2 Adherent Cultured Mø (5 µl/ml Ethanol for 18 hours).

3 Adherent Cultured Mø (50 µM Cycloheximide for 18 hours).

4 Suspension Cultured Mø (50 µM Cycloheximide for 18 hours).

5 Suspension Cultured Mø (5 µl/ml Ethanol for 18 hours).

6 Suspension Cultured Mø (50 µM Cycloheximide for 5 minutes).

7 Suspension Cultured Mø (50 µM Cycloheximide for 2 hours).

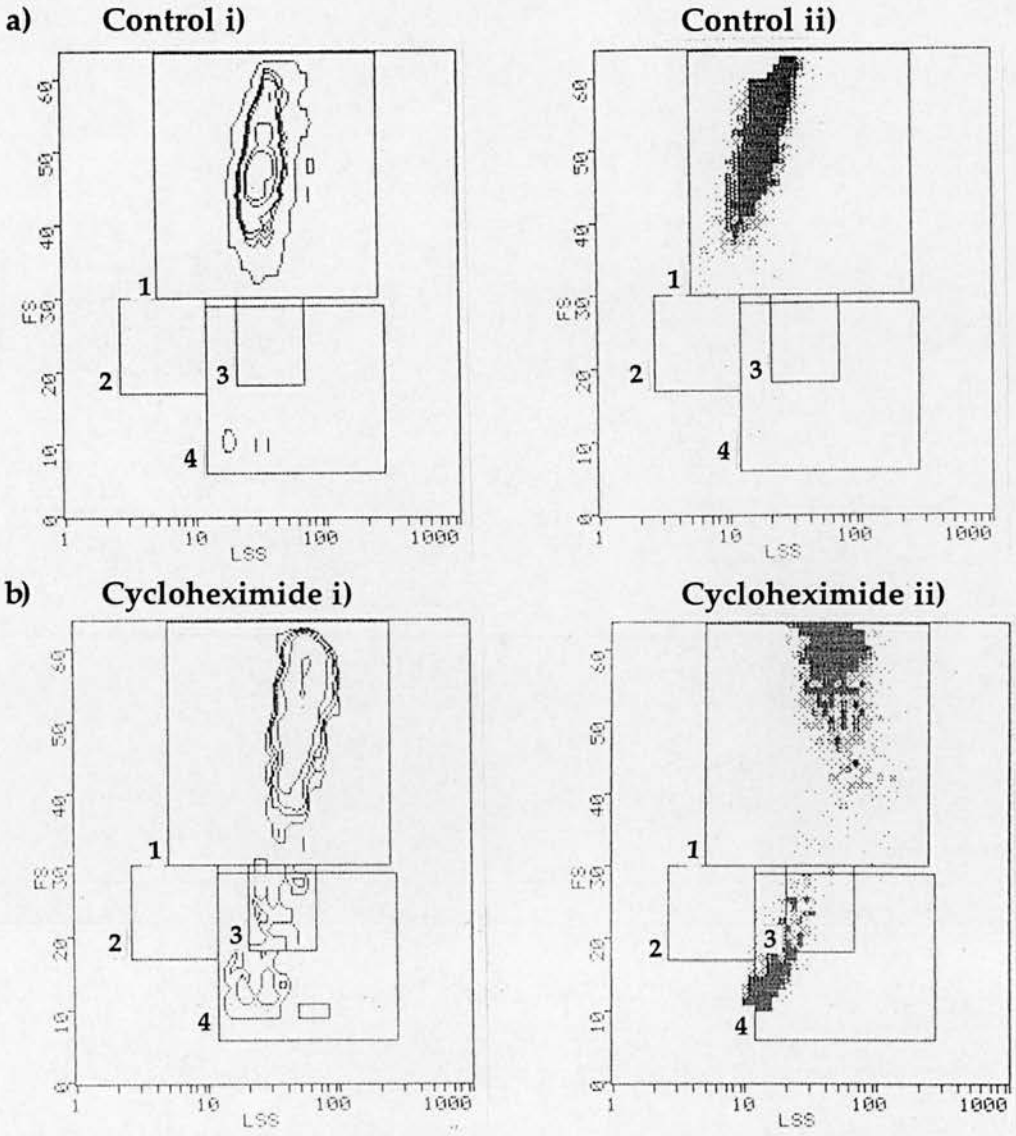
8 Suspension Cultured Mø (50 µM Cycloheximide for 8 hours).

9 Suspension Cultured Mø (50 µM Cycloheximide for 18 hours).

10 Suspension Cultured Mø (50 µM Cycloheximide for 40 hours).

11 Aged PMN with 55% apoptotic cells as positive control.

Figure 3. 7 Flow Cytometric Analysis of Apoptotic Macrophages.



Legend for Figure 3. 7 Forward and side scatter parameters of **a)** control Mø and **b)** cycloheximide treated Mø from two different donors - donors **i)** (contours) and **ii)** (dots). Acridine orange fluorescent microscopy confirmed there were <1% apoptotic Mø in the control cultures whilst after cycloheximide treatment (50μM for 14 hours), 21.5% of Mø from donor i) and 21% of Mø from donor ii) were apoptotic. This mirrored flow cytometric results with 1.1% and 0.8% Mø in apoptotic Mø gate in the control cultures but 18.1% and 30.9% in cycloheximide treated Mø. Gates: 1) Mø, 2) Lymphocytes, 3) PMN, 4) Apoptotic Mø.

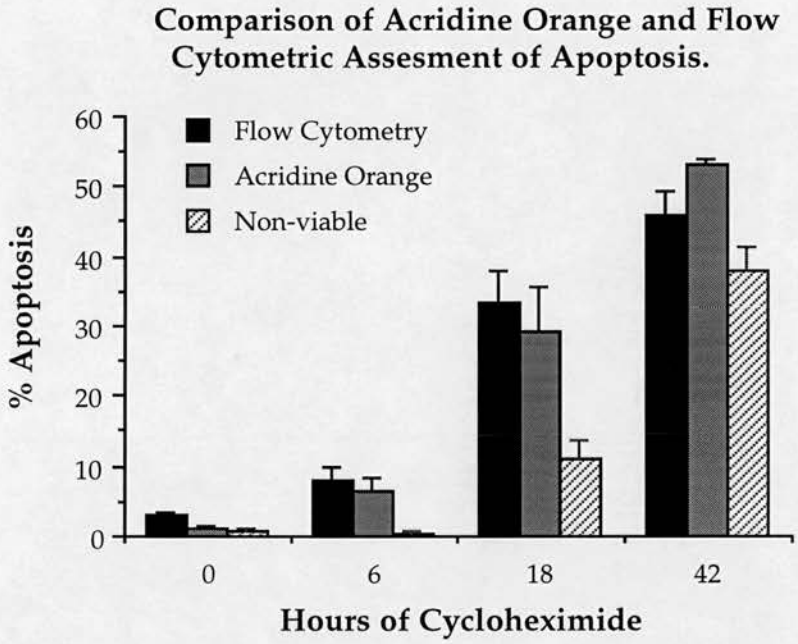
3. 2. 5. 2 Morphological Characteristics and Time Course.

As described in section 2. 7. 2. 1, monocytes and Mø had distinct forward and side scatter characteristics when examined by flow cytometry, allowing definition of a monocyte /Mø gate (Mø Gate) encompassing these cells (**Figure 2. 2**). The addition of cycloheximide (50 μM) to suspension cultures of Mø was shown to induce forward and side scatter changes indicating a loss of volume in a proportion of the Mø population which was not seen for cells treated with ethanol or medium alone (**Figure 3. 7**). Likewise a gate encompassing Mø with reduced cell volume after exposure to cycloheximide was created (Apoptotic Mø Gate) (see section 3. 2. 8 for validation that these were apoptotic Mø). Cells exposed to ethanol or medium alone had only 3.2% ± 0.6% (n = 8) cells within this Apoptotic Mø Gate after 18 hours (**Figure 3. 7a**), whereas 33.3% ± 4.8% (n = 8) of cells exposed to cycloheximide fell within this gate at 18 hours (**Figure 3. 7b**), the percentage of cells in the Apoptotic Mø Gate increasing with time after exposure to cycloheximide as summarized in **Figure 3. 8**.

In the same 8 experiments listed above, fluorescent microscopic analysis revealed that 29.3% ± 6.3 of cells were apoptotic at 18 hours in the cycloheximide treated group whilst 2.2 ± 0.3% of untreated cells had an apoptotic morphology. The percentage of apoptotic cells determined morphologically by acridine orange increased with time, paralleling the changes determined by flow cytometry as shown in **Figure 3. 8**. It is noteworthy that there was a significant increase in the number of non-viable cells concomitant with the induction of apoptosis but the morphological count of necrotic cells was less than 0.1%. Thus, unlike PMN where very few of the apoptotic PMN are trypan blue positive

(Haslett, 1994), apoptotic Mø do not retain the ability to exclude vital dyes for long.

Figure 3. 8

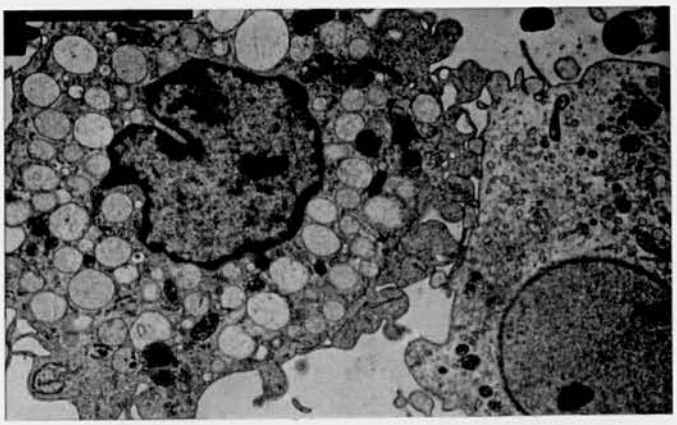


Legend for Figure 3. 8

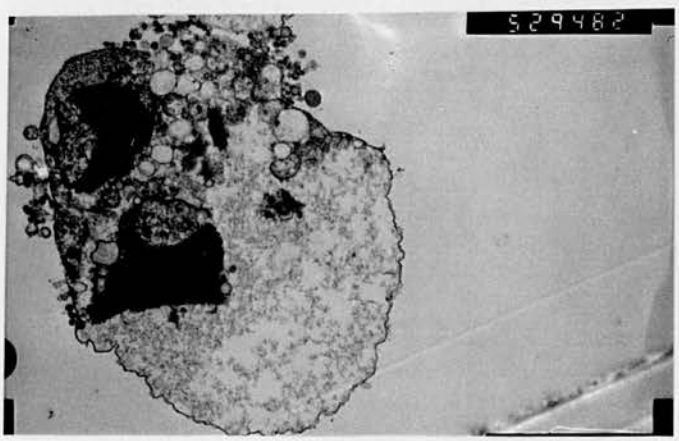
Cycloheximide (50 μM) was added to suspension cultures of human Mø, the percentage apoptosis was determined over time using acridine orange fluorescent microscopy and by flow cytometry using forward and side scatter. The percentage non-viable cells estimated by trypan blue exclusion are shown for the same time points, these are significantly less than the percentage apoptotic cells ($p<0.05$) for the 6 and 18 but not the 42 hour time points. Mø exposed to an equal volume of ethanol or medium alone were included as controls where the percent apoptosis was never greater than 5% at any time point, assessed by either method (Mean ± SE, n = 8).

Figure 3. 9 Electron Microscopy of Apoptotic Macrophages.

i) Non-Apoptotic Macrophages From Control Cultures.

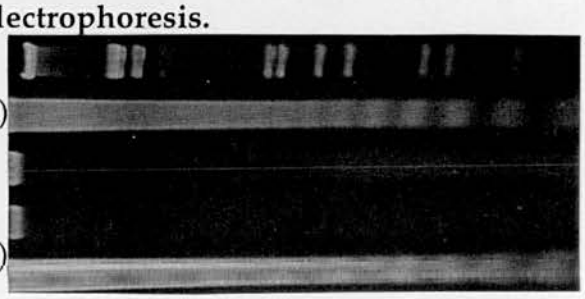


ii) Apoptotic Macrophage From Cycloheximide Treated Cultures.



iii) Corresponding DNA Gel Electrophoresis.

1 DNA size marker.
2 Mø + Cycloheximide (Donor 1)
3 Mø + Ethanol (Donor 1)
4 Mø + Ethanol (Donor 2)
5 Mø + Cycloheximide (Donor 2)



Legend for Figure 3. 9 Human Mø from two donors exposed to ethanol (control) or cycloheximide (50 μM) for 18 hours. Mø exposed to ethanol all had normal electron microscopy characteristics whilst many of the cycloheximide treated Mø demonstrated typical electron microscopic appearances of apoptosis with condensed nuclei and loss of cell volume; apoptosis was confirmed by DNA electrophoresis showing laddering.

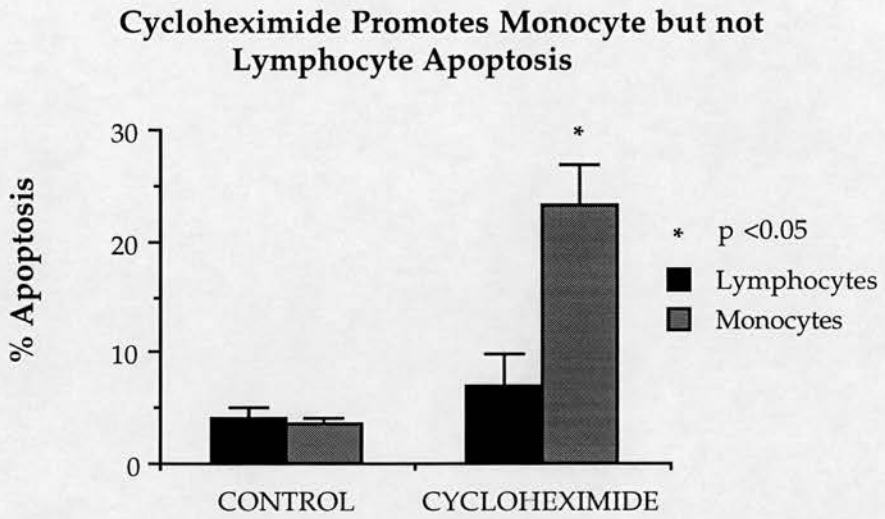
3. 2. 5. 3 Electron Microscopic Analysis of Macrophage Apoptosis.

In two experiments, electron microscopy was compared with acridine orange fluorescent microscopy counts of apoptosis and DNA gel electrophoresis findings. Suspension cultures of human Mø (94.4 and 96% pure) were treated with either ethanol or with 50 μM cycloheximide and incubated for 18 hours. Cycloheximide treatment resulted in 24 and 32% apoptosis as assessed by acridine orange fluorescent microscopy compared with only 1.2 and 4.4% apoptotic cells in untreated controls. Both of the cycloheximide treated populations exhibited a typical ladder pattern on DNA gel electrophoresis, whereas the control populations did not. In the cycloheximide treated population many cells exhibited characteristic electron microscopic features of apoptosis, including dense chromatin aggregation and cytoplasmic vacuolation (**Figure 3. 9**), none were seen in the controls.

3. 2. 5. 4 Effect of Cycloheximide on Lymphocytes.

Lymphocytes represented between 2 to 10% of the mononuclear population under investigation, depending on the preparative method used. To exclude the possibility that apoptotic lymphocytes could be contributing to the counts of apoptotic cells, the effect of cycloheximide on lymphocyte survival was investigated. In 3 separate experiments, non-adherent lymphocyte rich cell populations were incubated in Teflon foils for 24 hours with 50 μM cycloheximide or an equivalent volume of ethanol and compared with monocytes isolated from the same donor. The percent apoptosis was determined for each cell type using acridine orange fluorescence microscopy and the results presented in **Figure 3. 10**.

Figure 3. 10



Legend for Figure 3. 10

Comparison of the percent apoptosis induced in monocyte enriched (92 ± 2% monocytes) and lymphocyte enriched (89 ± 1% lymphocytes) populations after incubation with cycloheximide (50 μM) for 24 hours. Cycloheximide induced a significant increase in apoptosis (determined by acridine orange fluorescence microscopy) only in the monocyte enriched cells ($p<0.05$). (Mean ± SE, n = 3).

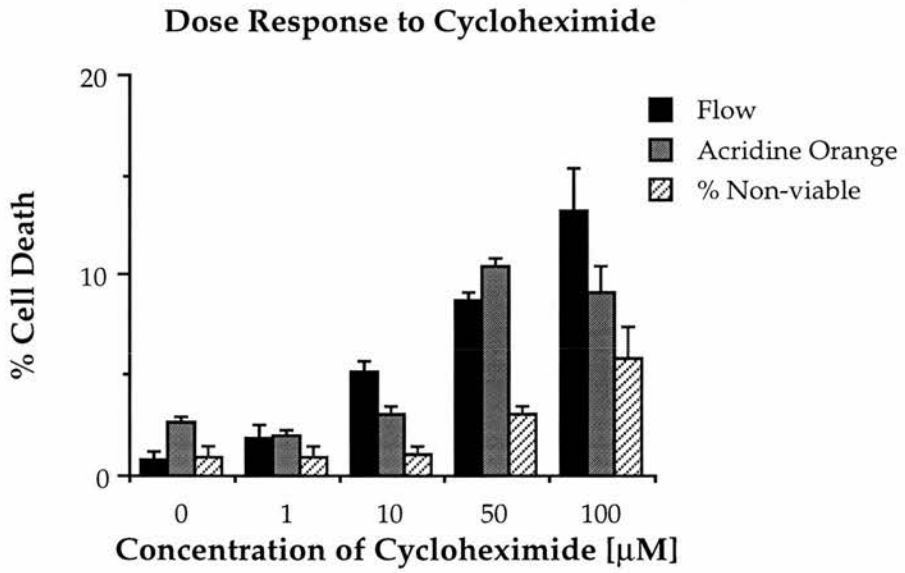
This shows that cycloheximide induces a 6.6 fold increase in the percent apoptotic cells in the monocyte rich population ($p<0.05$) whilst only a 1.6 fold increase in the lymphocyte rich population (p = n/s), indicating that cycloheximide does not significantly promote lymphocyte apoptosis. Lymphocyte preparations initially contained 11 ± 1% monocytes. After 24 hours there were 9 ± 0.4% non-apoptotic monocytes in the controls but only 3.5 ± 0.4% in the cycloheximide treated population, raising the possibility that apoptosis in the contaminating monocytes may partially account for the small rise in the percentage of apoptotic cells in the lymphocyte enriched population. There was no significant increase in

the percentage of apoptotic cells in lymphocyte enriched populations cultured for 72 hours (data not shown). This would be consistent with previously published data showing that cycloheximide does not promote lymphocyte apoptosis (Wyllie, 1984; Shi, 1990).

3. 2. 6 Dose Response for Induction of Apoptosis in Macrophages by Cycloheximide.

Using acridine orange as the standard method to quantify apoptosis together with confirmation by flow cytometry, the dose response of cycloheximide (1 - 500 μM) for induction of Mø apoptosis was determined. The percent apoptosis was assessed after 14 hours and the results shown in **Figure 3. 11**.

Figure 3. 11



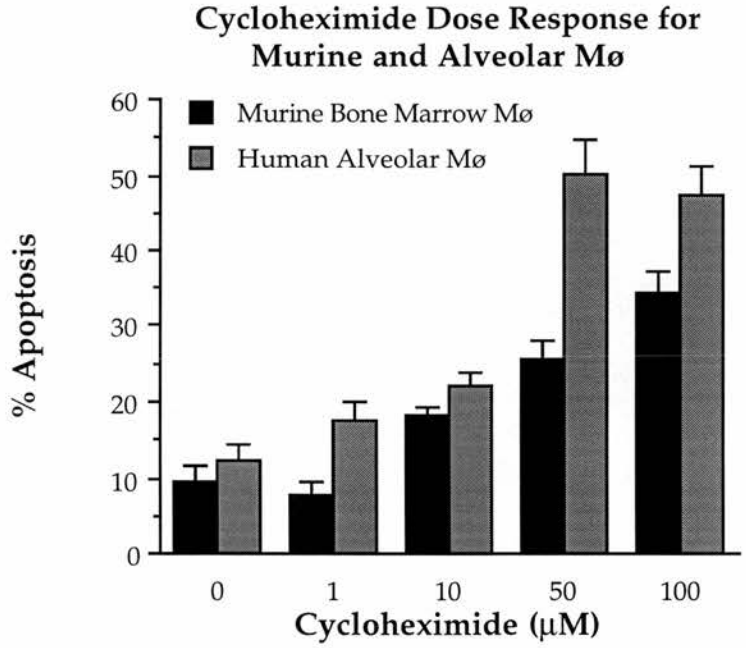
Legend for Figure 3. 11

Suspension cultures of human Mø were incubated with increasing concentrations of cycloheximide or an equivalent volume of ethanol as control (Cycloheximide 0 μM). After 14 hours, the percentage apoptosis was determined morphologically by acridine orange

fluorescence microscopy and by flow cytometry and viability by the ability to exclude trypan blue, results are expressed as mean ± SE, n = 5. There was no significant difference between percentage apoptosis as assessed morphologically or by flow cytometry. At concentrations of 10 and 50μM cycloheximide the percentage apoptotic cells were significantly greater than the non-viable cells. In two separate experiments - not plotted - 500 μM cycloheximide was also used, where 70.2 ± 8.9 % cells were apoptotic by flow cytometry, 61 ± 11% by acridine orange, 51 ± 4.9% cell were non-viable.

Cycloheximide exerted similar pro-apoptotic effects upon alveolar Mø and murine bone marrow derived Mø as shown in **Figure 3. 12**.

Figure 3. 12



Legend for Figure 3. 12

Suspension cultures of 7 day murine bone marrow derived Mø and human alveolar Mø were incubated with increasing concentrations of cycloheximide or with 10 μl ethanol (equivalent to the maximum

volume cycloheximide added) as control (Cycloheximide 0 μM). Percent apoptosis was determined morphologically by acridine orange fluorescent microscopy after 18 hours. In control cultures 4.3 ± 2.0% of murine bone marrow Mø and 10.8 ± 2.2% of alveolar Mø were non-viable as determined by trypan blue exclusion, this increased to 6.3 ± 4.2% and 17.8 ± 3.4% for 50 μM cycloheximide and 10 ± 3.5% and 27 ± 3.1% for 100 μM cycloheximide. Mean ± SE, n = 4 for each cell type.

3. 2. 7 Effect of Cycloheximide as a Protein Synthesis Inhibitor.

An experiment was performed to confirm that cycloheximide, at the concentrations used in this project, was acting to inhibit protein synthesis in Mø; the methods are detailed in section 2. 8. Briefly, Mø were washed in methionine free medium, ^{35}S methionine added and 4 x 10^6 Mø in methionine free medium were placed into Teflon foils, containing either 5 or 50 μM cycloheximide or ethanol as control. The cells were harvested after 20 hours incubation and the percentage apoptosis counted by acridine orange fluorescence microscopy. Mø were washed 3 times and the protein pellets precipitated, these fractions were counted in a beta counter to assess the incorporation of label as a measure of new protein production, the results are shown in **Table 3. 3**. Incubation of Mø in 50 μM cycloheximide resulted in almost total inhibition of protein synthesis, this was the dose that was subsequently used in all experiments. Since this experiment required a large number of cells (12 x 10^6 Mø, corresponding to an initial 28 - 34 x 10^6 monocytes), necessitating a large volume of blood (300 mls) from a single donor, this experiment was performed only once.

Table 3. 3

Protein Synthesis Inhibition Induced by Cycloheximide.

	Cells	Protein Pellets	
	% Apoptosis	cpm	Counts as % control
Control	3	49179	100
5 μM cycloheximide	5	8452	17
50 μM cycloheximide	60.5	945	1.9

	cpm		
Wash	Wash 1	Wash 2	Wash 3
Control	2 726 624	58 256	158 477
5 μM cycloheximide	3 219 768	112 003	20 137
50 μM cycloheximide	3 195 009	80 620	13 937

3. 2. 8 Flow Cytometric Separation of Apoptotic and Non-Apoptotic Cells.

Cycloheximide treated Mø were separated on the basis of cell size using a Becton Dickinson Cell Sorter. Three experiments were performed where Mø were treated with 50 μM cycloheximide for 18 hours yielding 18.1, 70.1 and 30.9% "shrunken" cells in the Apoptotic Mø Gate and 21.5, 63 and 21% apoptotic cells by acridine orange counts respectively. After sorting Mø on the basis of forward and side scatter into normal size and shrunken cells, the percentage of apoptotic cells in the Mø Gate and apoptotic Mø Gate on the EPICS II flow cytometer were determined and related to the percentage of apoptotic cells determined using acridine orange fluorescence microscopy, results are shown in **Table 3. 4.**

Table 3. 4

Acridine Orange and Flow Cytometry Counts of Macrophage Apoptosis Parallel Each Other.

	% Apoptotic Cells in Mø Gate		% Apoptotic Cells in Apoptotic Mø Gate	
Experiment	A.O.	Flow	A.O.	Flow
a	4.4	5.5	100	95.3
b	42.8	53.6	97.4	96.6
c	10	8.1	67	61.3

Legend for Table 3. 4

Results from three separate suspension cultures of human Mø from different donors, exposed to cycloheximide for 18 hours and sorted on the basis of cell size using a Becton Dickinson Cell Sorter. The % cells morphologically apoptotic when stained with acridine orange (AO) were compared with the % cells falling into the Mø Gate and the Apoptotic Mø Gate determined by forward and side scatter parameters on a Coulter EPICS II flow cytometer.

Hence the percent cells in the Apoptotic Mø Gate closely paralleled the acridine orange count of apoptotic cells. To confirm that these cells were indeed Mø, their expression of HLA-DR was determined. Relative to the non-binding control mAb MOPC-21C, 96.3% of cells from within the Mø Gate and 86% within the Apoptotic Mø Gate were HLA-DR positive, with a mean fluorescence of 120 and 28.4 respectively.

Gel electrophoresis of the DNA extracted from the sorted population confirmed that there was DNA fragmentation in those cells falling in the Apoptotic Mø Gate. In contrast there was only a very weak ladder pattern in the normal size cell population (Mø Gate), consistent with the low percentage of apoptotic cells remaining.

3. 2. 9 Surface Molecule Changes Associated with Macrophage Apoptosis.

The effect of apoptosis on the expression of 4 key Mø surface markers, CD11a, CD11b, CD14 and HLA-DR was determined, relative to that of the non-binding MOPC-21C control mAb. Since apoptotic and non-apoptotic Mø could be distinguished on the basis of forward and side scatter using flow cytometry, the relative binding of different mAb to non-apoptotic and apoptotic cells could be determined flow cytometrically by specific gating. Monocyte expression of these markers was determined and compared with Mø from the same donors, matured in Teflon foils over 5 days. These Mø were treated with 50 μM cycloheximide for 14 hours or an equivalent volume of ethanol as control. The effect of cycloheximide induced protein synthesis inhibition on Mø was controlled for by determining the surface expression on non-apoptotic Mø which had been exposed to cycloheximide for the same length of time as those that were apoptotic and comparing this with control Mø not exposed to any cycloheximide. The results are displayed in **Table 3. 5**, demonstrating that there is a decrease in the expression of CD11a, CD11b and CD14 on Mø relative to that of monocytes whilst the expression of HLA-DR increased during maturation. Although apoptotic Mø showed significant loss of expression of all 4 molecules compared with cycloheximide treated non-apoptotic Mø, the almost total (96.1%) loss of CD14 was far greater than

that of any of the other molecules tested (63.4% for HLA-DR, 68.1% for CD11a and 80% for CD11b).

Table 3. 5

Expression of Macrophage Surface Markers During Differentiation and Apoptosis.

		Macrophages		
Surface Marker	**Monocytes**	**Control**	**Non-Apoptotic CHX treated**	**Apoptotic CHX treated**
CD14	111 ± 5.1	42.9 ± 6.7§	23.4 ± 4.1¶	1.7 ± 0.2**
HLA-DR	25.7 ± 2.1	93.9 ± 17.6#	96.4 ± 18.7	35.1 ± 12.3*
CD11a	63.5 ± 4.4	9.6 ± 2.0§	11.6 ± 2.2	3.7 ± 0.5*
CD11b	68.1 ± 5.7	9.2 ± 4.3§	14.5 ± 3.5	2.9 ± 0.5*

§ Significant decrease from monocytes ($p < 0.01$)

Significant increase from monocytes ($p < 0.01$)

¶ Significant decrease in expression due to cycloheximide alone ($p<0.05$)

* Significant decrease in expression due to apoptosis ($p<0.01$)

** Significant decrease in expression due to apoptosis ($p<0.0005$)

Legend for Table 3. 5

Change in expression of CD11a, CD11b, CD14 and HLA-DR during monocyte to Mø maturation and the further changes associated with

cycloheximide induced apoptosis. The effect of cycloheximide alone is controlled for by determining the expression of the surface molecules on non-apoptotic Mø exposed to cycloheximide for the same period of time as those that undergo apoptosis. Results are expressed as mean fluorescence relative to that of a non-binding control mAb, MOPC-21C. Results are sequential data from 19 individuals, expressed as mean ± SE.

This loss of CD14 seems to be a relatively early event during the apoptotic process as continued culture of these apoptotic cells for a further 8 hours allowed further loss of expression of the integrins CD11a, CD11b and CD18 whilst CD14 had all been shed before the first determination of surface molecule expression occurred (data not shown).

3. 2. 10 Investigation of Phagocytosis in Macrophage Cultures.

Glucosamine has been shown to inhibit Mø uptake of apoptotic PMN (Savill, 1989b). To investigate whether it could prevent Mø phagocytosis of apoptotic Mø (i.e. any intrinsic "cannibalism"), cells were incubated for 14 hours in standard medium with added glucosamine (10 mM), glucosamine (50 mM) or an equivalent volume of PBS (100 µl) as control and the percent apoptotic cells then determined morphologically (n = 3). Inclusion of glucosamine had no significant effect on the percentage of apoptotic Mø at 14 hours, occasional phagocytosis was still observed and cell recoveries were identical between treatments. To confirm inhibition of uptake of apoptotic cells, a proportion of Mø from one of the experiments were incubated with aged PMN (65% apoptotic) in the presence and absence of glucosamine (50 mM) for 30 minutes. Glucosamine reduced the percentage of phagocytosis from 28.7% and

25.1% in the 2 control wells to 20.4% and 19.2% in the glucosamine treated wells, approximately a 24% inhibition in this experiment. Glucosamine had no effect on Mø phagocytosis of opsonized zymosan.

None of the documented inhibitors of phagocytosis of apoptotic cells (including amino sugars, blocking mAb to αvβ3 and CD36) produce complete inhibition of uptake (Savill, 1989b, 1990 and 1992). Even if the bulk of clearance of apoptotic Mø was by phagocytosis, to try and assess this using only partial inhibitors is unsatisfactory. Unless the rate of clearance of apoptotic cells by the remaining uninhibited Mø was previously saturated and could not be increased, any effect of a partial inhibitor could be obscured by increased phagocytosis by the remaining functional Mø.

To address the question in a different manner, Mø were labelled with fluorescent microspheres and apoptosis induced by cycloheximide. The aim was to separate apoptotic cells using a cell sorter and then quantify the ingestion of apoptotic Mø by their non-apoptotic counterparts. Technically this proved impossible due to release of significant quantities of the microspheres from "fragile" apoptotic cells.

The increase of apoptotic Mø in the presence of cycloheximide (section 3. 2. 5) suggested that although phagocytic clearance undoubtedly occurs it does not account for the bulk of apoptotic Mø. The presence of "ghost cell" bodies in monocyte cultures suggests that not all apoptotic cells are phagocytosed and they can instead continue to disintegrate in culture progressing towards secondary necrosis as described by Arends *et al.* for thymocytes (Arends, 1990). In view of these technical constraints, the

poor inhibition achieved with glucosamine and the lack of information on the time course of inhibition of phagocytosis and of any specific inhibitors capable of total blockade of apoptotic Mø uptake, this line of investigation was not pursued further.

3. 3 DISCUSSION

In this chapter I have shown that human monocytes can be isolated in sufficient quantity and purity by both density gradient centrifugation and by CCE and that these cells can be successfully matured into Mø either in suspension cultures in Teflon foils or by adherence to tissue culture plastic. The yields and purity were slightly greater from elutriation prepared monocytes but due to insoluble problems with cell viability during *in vitro* culture this preparative method had to be abandoned.

There was a steady attrition of monocyte numbers during maturation into Mø and to maximize Mø yields, the ideal starting concentration of monocytes in culture was shown to be 1×10^6 cells/ml. Over 5 days in culture nearly 55% of monocytes were lost, approximately 11% daily. Approximately 4.3% apoptotic monocytes were seen at any one time which suggested that apoptotic monocytes last for only 8 to 10 hours (see Appendix 1). Multinucleate giant cell formation, an uncommon event in suspension culture, did not account for the observed decline in cell numbers. The morphological counts also confirmed that cell necrosis, at less than 0.1% could not explain this loss either, especially as data from the freeze/thaw experiments (section 3. 2. 4) demonstrated that necrotic Mø had a life span of several hours. The greater than 40 fold excess in apoptosis over necrosis suggested that cells died by apoptosis whilst the lack of correlation between the percent apoptosis seen at any one time

and loss of cells demonstrated by total counts indicated that the apoptotic cells were short lived. A similar rate of loss was not observed for Mø.

The mechanism underlying the disappearance of these cell bodies rather than their persistence and accumulation needs to be explained. Certainly after the first 3 days in culture the cells have gained phagocytic ability and this could explain the disappearance of the apoptotic cells. In support of this was the fact that non-apoptotic Mø could be seen to contain apoptotic bodies with reasonable frequency (**Figure 3. 5**). This is not an isolated observation, Mø phagocytosis of apoptotic Mø has now been demonstrated by Khan *et al.* (Khan, 1993). Interestingly, results from three experiments suggest that glucosamine, an inhibitor of interaction of Mø with apoptotic PMN (Savill, 1989b), did not increase the percentage of apoptotic Mø observed in culture, although there are a number of problems with interpretation of this data, as discussed in section 3. 2. 10.

In the *in vitro* system described here, the persistence of the apoptotic body may be limited not because of phagocytosis but because of fragility. Certainly during the first 3 days in culture, prior to monocytes gaining phagocytic capacity, a different mechanism is needed to explain the disappearance of cells. An alternative explanation would be a relatively short life-span for the apoptotic monocyte. Unlike populations of apoptotic PMN, 95% of which retain the ability to exclude the vital dye trypan blue for 20 hours after the onset of significant apoptosis (Savill, 1989a), apoptotic monocytes and Mø are not so resilient. Although after 18 hours incubation with 50 μM cycloheximide the percentage Mø apoptosis was significantly greater than the percentage trypan blue positive cells, at other times and with other concentrations of

cycloheximide, the percentage monocytes and Mø that were trypan blue positive was often 50% or more of that of the percent apoptotic cells (see **Figures 3. 8** and **3. 11**). This suggests a greater membrane fragility of apoptotic monocytes and Mø than that of apoptotic PMN. This fragility was further demonstrated on cytospin preparations. Apoptotic PMN are relatively robust cell bodies which withstand the process of cytocentrifugation, which was not the case for apoptotic monocytes and Mø which usually fragmented completely and were poorly demonstrated by cytospin, hence the requirement for morphological counts of apoptosis by the relatively gentle method of acridine orange fluorescent staining. Finally, when acridine orange stains of 8 well slides of monocytes were viewed over the first 72 hours, indistinct bodies could be seen that did not contain any nuclear material (**Figure 3. 4**). These "ghost cells" were fragile and disrupted during resuspension. Others have reported trypan blue positive bodies without any defined nuclear or cytoplasmic structure in myeloid cell lines treated with cytokines to induce apoptosis (Lotem, 1992). Furthermore cycloheximide treatment of monocytes not only increased the numbers of apoptotic cells but with time progressively smaller apoptotic nuclear fragments and increasing numbers of "ghost cells" were seen (**Figure 3. 4iii**). Together these data suggest that "ghost cells" are fragile late monocyte apoptotic bodies that have extruded all nuclear material. It seems most likely from the above considerations that monocytes undergo constitutive apoptosis at a rate of approximately 0.5% per hour in the presence of serum and that apoptotic monocytes are short lived relatively fragile bodies. During the early stages of monocyte culture, apoptotic cells progressively lose their nuclear material, remaining only as intact apoptotic cells for 8 - 10 hours thereafter becoming indistinct "ghost cell" bodies, thus only approximately 4% of

apoptotic cells will be observed in any single "snapshot" of these cells. Later when Mø gain phagocytic competence they may cannibalize the apoptotic cells although the relevance of this route of clearance is still unclear. For this reason I used both morphologic counts of apoptosis and estimates of total cell number to determine cell loss.

The protein synthesis inhibitor cycloheximide consistently induced apoptosis in monocytes and Mø, providing a reproducible model for the study of Mø apoptosis. This finding is in accord with that of Waring who demonstrated that the protein synthesis inhibitors ricin and cycloheximide, as well as actinomycin D all induced apoptosis in murine Mø (Waring, 1990). Cycloheximide is known to also induce apoptosis in PMN (Haslett, 1994) but to retard the rate of apoptosis in thymocytes (Wyllie, 1984). Cycloheximide also increased the percent apoptotic cells in monocyte enriched cultures from 3.5 to 23.2% but in lymphocyte enriched cultures apoptosis was only increased from 4 to 7% and this increase was considered to represent increasing apoptosis among contaminating monocytes. The differential effect of cycloheximide on the induction of apoptosis in cells is of interest mechanistically as it suggests that distinct cell populations have different controls on the induction of apoptosis.

Monocyte and Mø apoptosis was confirmed by a number of methods including the two "gold standards" - electron microscopy and DNA electrophoresis. However for rapid quantification alternative methods were sought. Acridine orange morphological counts and flow cytometric determination of apoptosis were shown to parallel each other and as there was little evidence of cell disruption they were suitable for routine use. Cell sorting techniques, verified by DNA gel electrophoresis

confirmed that both acridine orange fluorescent microscopy and flow cytometry were accurate and reproducible methods for determining apoptosis in monocyte and Mø populations.

Other investigators have used a number of different methods to assess cell survival, viability and cell numbers. The direct cell count of fixed cells conforms with the methods of Johnson *et al.* , whilst Mangan *et al.* used propidium iodide (PI) staining of DNA to determine cell viability and apoptosis (Johnson, 1977; Mangan, 1991a). It is unlikely that PI staining would distinguish "ghost cells", as they do not have any nuclear material as demonstrated by acridine orange staining. I investigated PI staining but found that the high background fluorescence of Mø populations, especially alveolar Mø, obscured changes associated with apoptosis. Furthermore, there was an increase in PI uptake with time during monocyte culture, making comparisons over time difficult.

Interestingly, CD14 was found to be extensively lost from the surface of apoptotic Mø. The significance of this observation was investigated by examining the changes in surface expression during the apoptotic process of a number of functionally important molecules. Despite reduction in expression during apoptosis of both the integrins CD11a and CD11b and in HLA-DR expression, the loss of CD14 was a much more pronounced event, being ten fold greater in magnitude. There may be interesting parallels with the loss of the GPI linked molecule CD16 on apoptotic PMN (Dransfield, 1994). Like apoptotic Mø, apoptotic PMN lose only specific surface molecules with apoptosis, notably apoptotic PMN also retain CD11b/CD18 expression (Dransfield, 1995). The loss of CD14 is unlikely to be due to any prior cellular activation, TNF, IL-1 and LPS

having all been shown to increase CD14 expression (Zeigler-Heitbrock, 1993). Despite the GPI linkage it seems that CD14 shedding does not occur by phosphatidyl inositol cleavage, rather it is by protease digestion (Bazil, 1991). The physiological relevance of this to the apoptotic process is unknown but it is of interest that this loss of CD14 seems to occur early in the apoptotic process. This may "functionally isolate" these Mø and thus act as a control on Mø responses, however soluble CD14 in combination with LPS binding protein may retain the capacity to present LPS to non-CD14 expressing cells (Haziot, 1993). Thus it is possible to argue that receptor shedding may both increase or decrease responses to LPS.

Hence in summary, a reliable system for isolation and culture of monocytes was established that allowed for their maturation into Mø. Exposure to 50 μM cycloheximide for 14 - 18 hours consistently induced apoptosis which could be easily quantified by acridine orange fluorescence microscopy and/or flow cytometry along with an estimate of total cell numbers. Acridine orange morphological counts were used routinely as many experiments included both suspension and adherence cultures, and acridine orange counts could be easily performed on the adherence cultures without disturbing the cells. Where suspension cultures alone were used, both methods of assessment were usually employed. With this essential background information relating to monocyte and Mø culture *in vitro* and preliminary characterization of the apoptotic process in these cells, I sought to investigate factors affecting monocyte and Mø apoptosis (detailed in Chapter 4 and 6) and to further characterize the changes in surface phenotype of these apoptotic cells (detailed in Chapter 5). The investigation of the *in vivo* relevance of Mø apoptosis during the resolution of inflammation is discussed in Chapter 7.

3. 4 Appendix 1

After 120 hours *in vitro* suspension culture (10% serum), monocytes numbers had declined linearly to approximately 46.5% of the starting number (see **Table 3. 2**). For a starting cell count of 1 x 10^7 cells this would represent a final recovery of 4.65 x 10^6 cells, or a loss of 5.35 x 10^6 cells over 120 hours, which - if this truly is a linear decline - represents a loss of 44 600 or 0.446% cells/hour. In the following table the life-span of an apoptotic monocyte was taken to be 10 hours. Using this assumption, the expected number of monocytes and corresponding percentage of apoptotic cells in culture are shown and are compared with the actual counts.

Time	**Total cells**	**Apoptotic cells**		*Actual Apoptosis*
(Hrs)	**(No.)**	**(No.)**	**(%)**	*(%)*
0	10 000 000	0	0	*2.1*
1	9 955 400	44 600	0.446	-
2	9 910 999	89 001	0.890	*4.4*
3	9 866 789	133 204	1.33	-
4	9 822 783	177 210	1.77	*4.1*
5	9 778 973	221 020	2.21	-
6	9 735 359	264 634	2.65	*4.4*
7	9 691 939	308 054	3.08	-
8	9 648 713	351 280	3.51	*4.2*
9	9 605 680	394 313	3.94	-
10	9 562 839	437 154	4.37	*4.0*
11	9 520 189	435 204	4.37	-
12	9 477 729	433 263	4.37	*4.6*
13	9 435 458	431 331	4.37	-
14	9 393 376	429 407	4.37	-

After 10 hours the initial 44 600 apoptotic cells will have disappeared (by disintegration or being eliminated by some other means) hence the total number of apoptotic cells will peak at this time and there after decline. Although new apoptotic cells will be added they will come from a smaller

pool of non-apoptotic cells. The percentage of apoptotic cells will remain constant however at 4.37% as the slowly declining pool of apoptotic cells remains a constant proportion of the slowly declining total number of cells. Using similar calculations it can be seen that if the life-span of apoptotic cells was longer, e.g. 20 hours then the percentage of apoptotic cells would steadily increase to 8.54% by 20 hours and then remain constant. Likewise if the half life was shorter e.g. 5 hours the percentage of apoptotic cells would plateau at 2.21% after this time in culture. Comparison of this calculation with the actual results obtained (shown in italics here - full results in **Table 3. 2**) demonstrate several things:

- The measured percentage of apoptotic cells was 4.3% at any single time point, so this calculation would suggest that the life-span of these cells was approximately 10 hours.
- The measured percentage of apoptotic cells differed from that expected using this simple calculation over the first few hours, if the life-span of the apoptotic cells was 10 hours. This calculation suggests that there would be a steady increase in the percentage of apoptotic cells to a maximum of 4.37%, however in reality the percentage of apoptotic cells was similar at 4, 6, 8, 10, 12 and 18 hours.
- This calculation assumes that there are no apoptotic cells at the start while infact approximately 2% of the cells were apoptotic at this time. Recalculating, starting with 2% apoptotic cells, still underestimates the true percentage of apoptotic cells over the first four hours.
- This suggests that although there is a relatively steady decline in cell number, the rate with which cells undergo apoptosis may not be constant. Initially there may be a small wave of apoptosis and thereafter a relatively constant number of cells become apoptotic and these apoptotic cells may well have a life-span of approximately 10 hours. This suggests, as would be expected, that human peripheral blood monocytes are unlikely to be a synchronous population but rather contain cells of differing ages and susceptibility to apoptosis.

Chapter 4

CYTOKINES AND SERUM SURVIVAL FACTORS IN MONOCYTE AND MACROPHAGE APOPTOSIS.

4. 1 INTRODUCTION.

Despite clear evidence that the life and death of cells are influenced by cytokines and survival factors in serum, the factors contributing to Mø longevity are unknown. It is well established *in vivo* that Mø survive for many days (van Furth, 1992) and this has also been shown for Mø in *in vitro* cultures (Helinski, 1988; Brugger, 1991). Cell survival is influenced by many factors, principally by cytokines and growth factors but also by cell - cell contact and cell - matrix interactions and *in vitro* by adherence (Khan, 1993). *In vitro* cultured Mø require serum for appropriate survival (Mangan, 1991a; Munn 1995) and differentiation (Brugger, 1991). The exact nature of the survival factor(s) in serum are unclear although certainly in the murine system CSF-1 is crucially important (Tushinski, 1982).

At the outset of this project there were only two reports on factors that could induce Mø death, both on murine Mø; ATP, cytotoxic T lymphocytes and protein synthesis inhibition (cycloheximide or actinomycin D) had been shown to cause Mø apoptosis whilst 20 mM hydrogen peroxide and freeze thawing caused necrosis (Waring, 1990; Hogquist, 1991a). These studies provided insight into mechanisms involved in the apoptotic process, demonstrating the importance of the protein synthetic machinery for Mø survival (Waring, 1990) and implicating a role for ICE during Mø apoptosis (Hogquist, 1991a). These studies did not address the issue of physiological controls on Mø survival and the mechanisms involved in Mø longevity. Mangan *et al.* demonstrated that monocytes rapidly undergo apoptosis in response to serum withdrawal and that this could be prevented by physiologically

relevant activating signals including LPS, TNF-α and IL-1 (Mangan 1991a and b). They proposed that monocytes may be short lived cells requiring continued stimulation to survive. This suggested to me that there may be a difference between monocyte and Mø survival characteristics.

If control of monocyte or Mø longevity represents an important mechanism through which inflammation is limited then one may postulate that cytokines could exert differential regulatory effects on survival of these cells. The early pro-inflammatory cytokines TNF-α and IL-1 would be expected to delay apoptosis, whilst the cytokines involved in the resolution stages of inflammation, such as TGF-β or IL-4 may be pro-apoptotic.

I therefore aimed to investigate the physiological controls of monocyte and Mø survival including the influence of serum, adherence, the effects of a range of cytokines and the relationship between these factors. To explore the possibility of a difference in monocyte and Mø survival characteristics I examined a range of cell types; the promonocytic U937 cell line, human monocytes, human monocyte-derived Mø, alveolar Mø, as well as murine bone marrow derived Mø. This spectrum of cells was chosen as differential sensitivities to potential regulatory factors might be expected to be revealed at different maturational stages.

4. 2 RESULTS.

4. 2. 1 U937 Cells Die Rapidly by Apoptosis in the Absence of Serum.

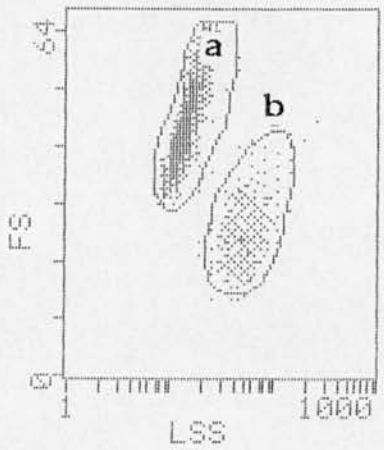
U937 cells, maintained in log phase growth, had a doubling time of approximately 2 - 3 days cultured in RPMI supplemented with 10% FCS

and 2 mM L-glutamine. When washed twice and resuspended at approximately 1 x 10^6 cells/ml these cultures contained 7 ± 1.8% (n = 4) apoptotic cells, determined both morphologically and by flow cytometry. This was confirmed by CCE separation of non-apoptotic and apoptotic cells (**Figure 4. 1**). As shown in **Figure 4. 2**, there was no significant increase in apoptosis over 6 hours when U937 cells were cultured in RPMI containing 10% FCS and L-glutamine. This contrasted with a huge rise in apoptosis within 1 hour when cells were cultured in RPMI plus L-glutamine alone (no 6 hour time point reported as extensive secondary necrosis invalidated morphological assessment of apoptosis and estimates of cell recovery). There was also a significant rise in the number of apoptotic U937 cells after 6 hours culture in IDMEM alone ($p < 0.01$, n = 4). Cell counts in RPMI with 10% FCS rose from 1.14 ± 0.08 at the start to 1.43 ± 0.06 x 10^6/ml after 1 hour and 1.57 ± 0.09 x10^6/ml by 6 hours. The corresponding count for RPMI at 1 hour was 0.48 ± 0.15 x 10^6 cells/ml ($p<0.05$, n = 4) and for IDMEM at 1 and 6 hours were 1.18 ± 0.05 x10^6/ml and 0.89 ± 0.08 10^6/ml respectively.

To determine whether altered pH accounted for this rapid death in RPMI, the pH of the media was determined at 1 hour and was 7.61, 7.60 and 7.74 for RPMI with 10% FCS, IDMEM and RPMI respectively, suggesting that there may be differences in the buffering capacity of the media that account for these findings. There was no further change in the pH of the medium by the 6 hour time period (7.59 and 7.60 for RPMI with FCS and IDMEM respectively) indicating that the later rise in the apoptosis levels in IDMEM may be a serum depletion effect.

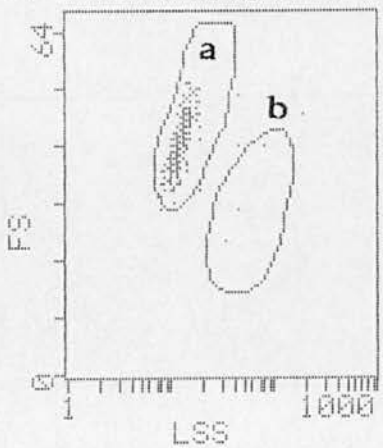
Figure 4. 1 Separation of Non-Apoptotic and Apoptotic U937 Cells.

i) Pre-Elutriation.

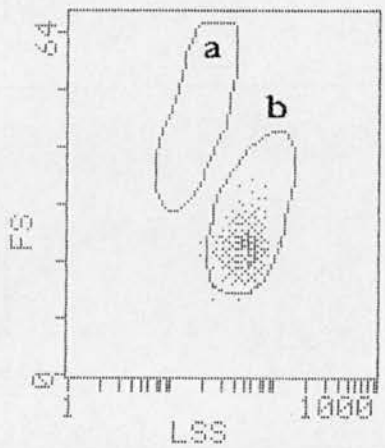


ii) Post Elutriation.

a) Non-Apoptotic.



b) Apoptotic.



iii) DNA Gel Electrophoresis.

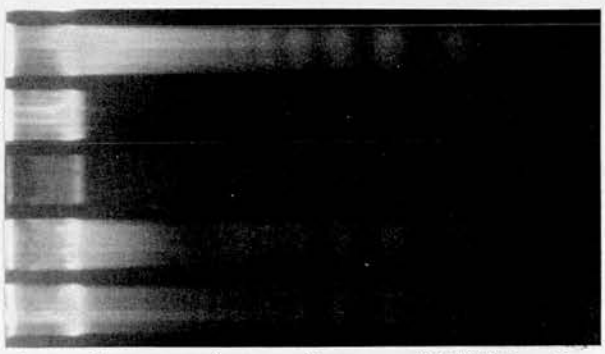
1 U937 Cells Pre-CCE

2 Post-CCE (a) Non-Apoptotic

3 N/A

4 Post CCE (b) Apoptotic

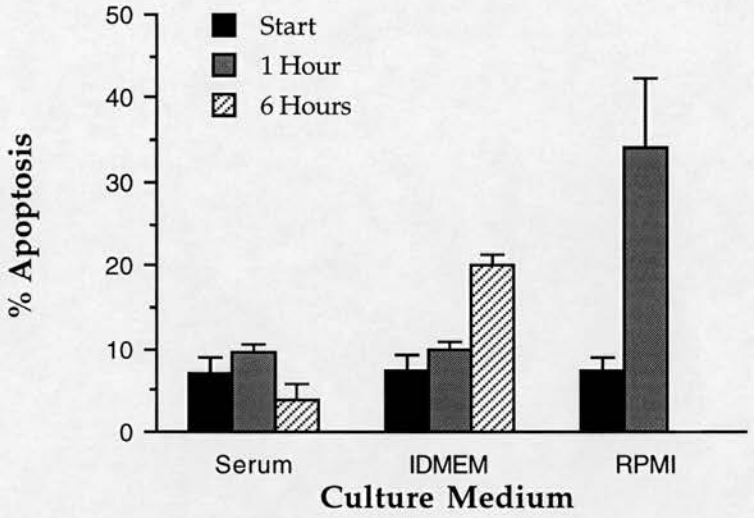
5 Post CCE (b) Apoptotic



Legend for Figure 4. 3 Flow cytometry of serum free cultures of U937 cells using forward and side scatter demonstrated 2 populations, these were separated by CCE and using both acridine orange fluorescence microscopy (not shown) and DNA gel electrophoresis, the larger cells (a) were shown to be non-apoptotic and the smaller cells (b) were shown to be apoptotic.

Figure 4. 2

Effect of Culture Medium on U937 Cell Apoptosis.



Legend for Figure 4. 2

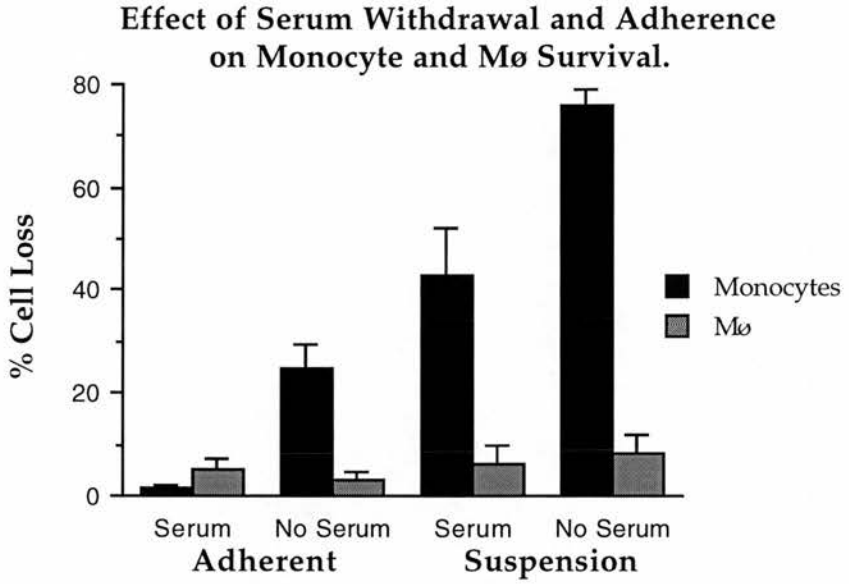
U937 cells were washed, resuspended at approximately 1 x 10^6 cells/ml and cultured in RPMI supplemented with 2 mM L-glutamine and 10% FCS (Serum), RPMI with 2 mM L-glutamine (RPMI) or IDMEM with 2 mM L-glutamine (IDMEM). Flasks were resuspended at either 1 or 6 hours when cells were counted, percentage apoptosis determined by acridine orange fluorescent microscopy and flow cytometry and the pH of the media measured.

4. 2. 2 Effects of Serum Withdrawal and Adherence on Monocyte and Macrophage Survival.

Serum withdrawal caused a significant loss of adherent cultured monocytes, with a 25 ± 4.3% reduction in number over 42 hours in culture, compared to only a 1.3 ± 1% reduction in the presence of serum ($p< 0.01$, $n = 6$) as shown in **Figure 4. 3**. After the initial 3 washes to remove serum, cells remained undisturbed until counted, hence this difference could not be attributed to cell detachment and loss during washes. Cell counts were performed by racking down through the depth

of the culture medium in order to detect non-adherent cells; there were very few floating cells in any of the wells.

Figure 4. 3



Legend for Figure 4. 3

Loss of human monocytes and Mø after 42 hours culture in the presence of 10% autologous serum (Serum) or medium alone (No Serum). Cells were cultured either adherent to 8 well slides or in suspension in Teflon foils, the percentage of the original number of cells remaining after 42 hours was estimated from changes in haemocytometer counts after resuspension of cells (for suspension cultures) and by counting the number of cells per high power field (HPF) (in adherent cultures). Results are sequential data for monocytes and Mø derived from 6 individuals and expressed as mean ± SE.

Figure 4. 3 also shows that the recovery of monocytes was lower when the cells were cultured in suspension, whether in the presence or absence of serum. Cell loss was significantly greater for monocytes cultured in the

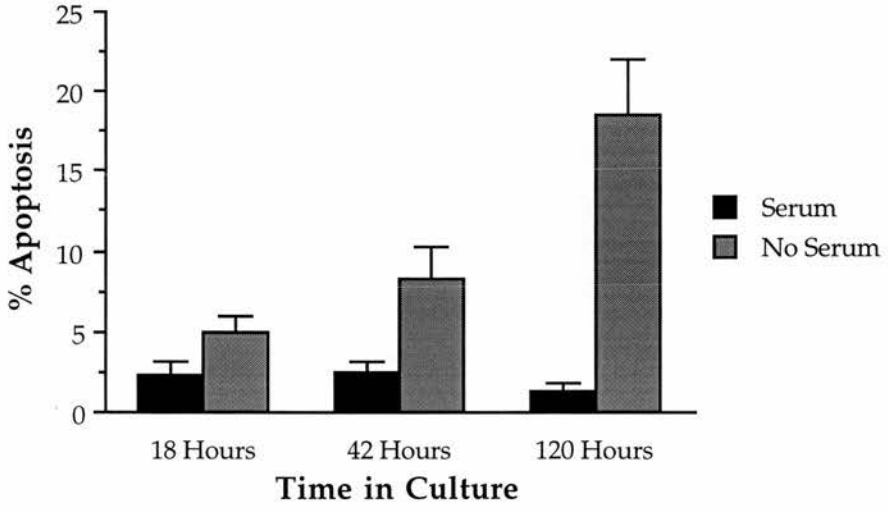
absence of serum (76 ± 3% reduction in cell number in the absence of serum compared with 43 ± 9% with serum, $p < 0.05$). In contrast to monocytes, culturing Mø in the presence or absence of serum, whether adherent or not, did not affect the number of cells recovered, as shown in **Figure 4. 3** there was no significant change in Mø cell number over the 42 hour time course, raising the possibility that there are differences in the regulation of apoptosis for monocytes and Mø.

4. 2. 3 Monocytes Die by Apoptosis in the Absence of Serum.

Figure 4. 4 demonstrates that adherent cultured monocytes had significantly higher levels of apoptosis in the absence than the presence of serum by 18 hours, this effect increasing at 42 hours and 5 days.

Figure 4. 4

Serum Withdrawal Promotes Monocyte Apoptosis.



Legend for Figure 4. 4

Percentage apoptosis in adherent monocytes cultured in the presence (Serum) or absence (No Serum) of 10% autologous serum in 8 well slides. Apoptosis determined by acridine orange fluorescent microscopy. Data are sequential results from 13 individuals expressed

as mean ± SE. At each time point there is a significantly greater percentage apoptotic cells in the absence than the presence of serum ($p<0.05$ at 18 hours, $p<0.01$ at 42 hours and $p<0.0001$ at 120 hours). In the absence of serum the percentage of apoptotic cells increases significantly at 42 hours ($p <0.01$) and again at the 120 hours ($p < 0.01$), in the presence of serum there is no significant change.

Comparison of the percentage apoptosis with the percentage of cell loss in the absence of serum (percentage apoptosis of 8.3 ± 1.9% and 18.5 ± 3.6% for 42 hours and 5 days whilst cell loss of 25 ± 4.3% and 76 ± 3.2% respectively) revealed that cell losses were greater at each time point than the recorded morphological levels of apoptosis. There was no morphological evidence of necrosis at any time and DNA electrophoresis confirmed the morphological findings of increased monocyte apoptosis in the absence of serum.

As noted earlier, there was no significant decrease in Mø recovery following 42 hours culture in the presence or absence of serum. Although there was a very small, statistically significant increased percentage of apoptosis in Mø cultured in the absence of serum (1.6 ± 1.2% vs. 2.2 ± 1.3% in the presence and absence of serum respectively), the low percentages did not affect cell numbers significantly. Monocytes had significantly greater levels of apoptosis after 42 hours in the absence of serum than Mø (8.3% vs. 2.2% $p<0.01$) and likewise for cell loss (25 ± 4.3 vs. 2.2 ± 1.3 $p < 0.001$).

4. 2. 4 Murine Bone Marrow Derived Macrophages Die Rapidly in the Absence of Serum.

Seven day murine bone marrow derived Mø were cultured in either standard medium (IDMEM, 25% FCS, 25% L 929 cell supernatant) or in IDMEM alone. After 6 hours there was a significant increase in apoptosis in those cells cultured in the absence of both serum and L929 supernatant, this difference was more marked by 18 hours when it was also reflected in changes in cell numbers. After 42 hours in culture marked differences in recovery were obvious but far less apoptosis was evident as shown in **Table 4. 1**. The addition of FCS but not CSF-1 partially prevented this cells loss suggesting that serum and CSF-1 were acting independently.

Table 4. 1

The Effect of Serum and CSF-1 on Murine Bone Marrow Derived Macrophage Survival Over 42 Hours.

	6 Hours		18 Hours		42 Hours	
	Apop (%)	Recovery (%)	Apop (%)	Recovery (%)	Apop (%)	Recovery (%)
Medium	14.2± 3**	100 ± 5	38.3 ± 10**	70 ± 5*	4.8 ± 1	34 ± 7**
FCS	-	-	-	-	2.0 ± .5	60 ± 6*
FCS + CSF-1	1.7 ± 1	100 ± 3	2.8 ± 2	110 ±4	0.3 ± .1	95 ± 3

Legend for Table 4. 1

Murine bone marrow derived Mø, seeded in Teflon foils at 0.375 x 10^6 /ml, were washed on day 5, resuspended at 1 x 10^6/ml, washed twice on day 7 and resuspended at 1.75 ± 0.3 x 10^6/ml in either IDMEM alone (Medium), IDMEM with FCS (FCS) or IDMEM, FCS and L929

supernatant (FCS + CSF-1). Percentage apoptosis was determined by acridine orange fluorescent microscopy and cell numbers by haemocytometer counts. Cell recoveries at 6, 18 and 42 hours are expressed as percentage of the initial cell number with 100% representing no change (n = 4). Comparison is made between FCS + CSF-1 and other treatments for each time point and significance denoted by * for $p<0.05$ and ** for $p<0.01$.

4. 2. 5 Alveolar Macrophages Die by Apoptosis in the Absence of Serum.

Culturing human alveolar Mø in suspension for 84 hours in the absence of serum resulted in a 30 ± 9% reduction in the number of cells recovered compared with a loss of only 10 ± 3 % in the presence of serum (n = 3). At this time point there were 27.1 ± 12% and 5.4 ± 1.2% apoptotic cells in the absence and presence of serum respectively. There was no difference in the amount of apoptosis at 18 hours: 8.95 ± 2.5% and 8.75 ± 2.25% in the presence and absence of serum respectively (n=8). Due to difficulties in recruiting volunteers to undergo bronchoalveolar lavage these numbers did not achieve statistical significance, however a trend towards increased apoptosis in the absence of serum was apparent.

4. 2. 6 Effect of Cytokines on Monocyte and Macrophage Apoptosis.

4. 2. 6. 1 Effect of TNF-α on Monocyte and Macrophage Apoptosis.

TNF-α at 1000 units/ml delays monocyte apoptosis in serum free medium (Mangan, 1991a) but induces apoptosis in PMN at a concentration of 100 to 1000 units/ml (Takeda, 1993; Tsuchida, 1995). Data presented below demonstrates that the addition of 200 -2000 units of TNF-α (approx. 10 - 100 ng/ml) did not alter the percent apoptosis seen for

either monocytes or Mø when cultured in the presence of serum. This was true for both suspension and adherent cultures. Results are summarized in **Table 4. 2**.

Table 4. 2

TNF-α does Not Alter Percentage of Apoptotic Monocytes or Macrophages in Culture.

	Apoptotic monocytes		Apoptotic macrophages	
	Suspension (%)	Adherence (%)	Suspension (%)	Adherence (%)
Control	4.9 ± 2.9	4.3 ± 2.6	1.7 ± 0.5	1.1 ± 0.4
TNF-α	4.1 ± 3.1	4.3 ± 2.1	1.3 ± 0.9	1.8 ± 0.8
n	3	4	3	6

Legend for Table 4. 2

Percentage of apoptotic monocytes or Mø after 42 hours suspension culture (seeded at 1 x 10^6/ml) or adherent culture (8 well slides) in the presence or absence of 2000 units/ml TNF-α. The results were the same for 200 and 800 units/ml of TNF-α (data not shown). There was no significant difference between any of the treatments.

As TNF-α exhibits an early (6 hour) apoptosis promoting effect and a later (20 hour) apoptosis preventing effect when added to PMN (Dr. E. Chilvers, personal communication), apoptosis counts were made at 6, 18

and 42 hours for both monocytes and Mø cultured in serum in 8 well slides to determine if the same pertained for these cells. The results, shown in **Table 4. 3**, demonstrate that TNF-α had no significant effect on apoptosis at any of these time points.

Table 4. 3

TNF-α has no Time Dependent Effect on Monocyte or Macrophage Apoptosis.

	% Apoptosis		
	6 hours	**18 hours**	**42 hours**
Monocytes			
Control	4.9 ± 0.9	3.7 ± 1.1	3.2 ± 1.1
TNF-α	4.2 ± 1.0	4.0 ± 0.9	5.1 ± 1.3
Macrophages			
Control	1.7 ± 0.6	1.9 ± 0.4	2.5 ± 1.0
TNF-α	1.9 ± 0.3	1.9 ± 0.5	2.4 ± 0.8

Legend for Table 4. 3

Percentage apoptosis for adherent cultures of monocytes and Mø in the presence or absence of 2000 units/ml TNF-α. Results are the means ± SE, n = 3, p = n/s. TNF-α does not affect cell recovery (cells/HPF) for cells cultured in the presence of serum.

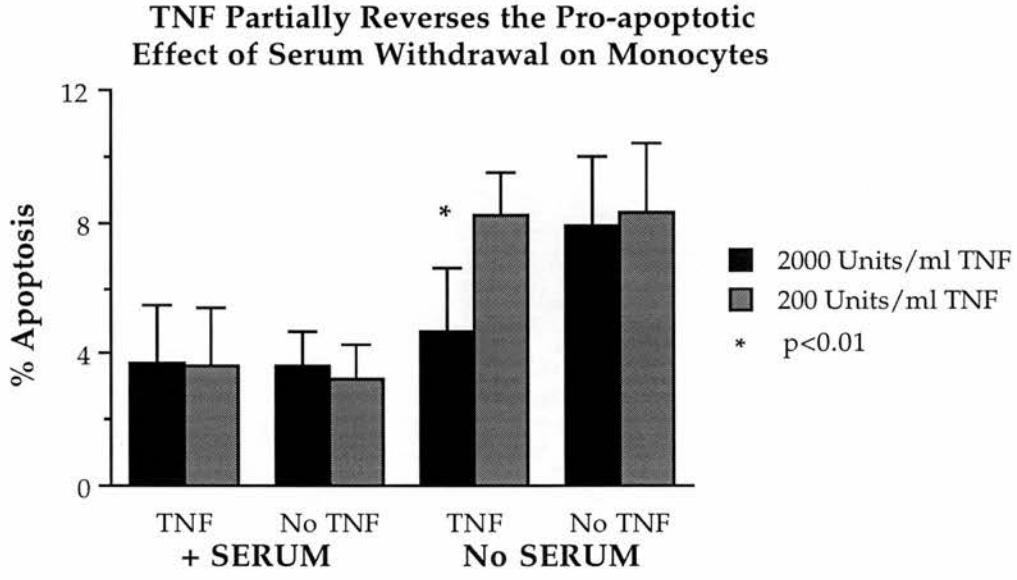
Examination of cell recovery after 5 days suspension culture showed no difference between control and TNF-α treated cells. Monocytes were

cultured at 1 x 10^6/ml in 10% autologous serum in the presence or absence of 2000 units/ml TNF-α and cell counts after 5 days were 0.46 ± 0.09 x 10^6/ml and 0.50 ± 0.10 x 10^6/ml respectively (p = n/s, n=3).

4. 2. 6. 2 TNF-α and Serum Withdrawal.

Figure 4. 5 shows that 2000 units but not 200 units/ml TNF-α significantly prevented the pro-apoptotic action of serum withdrawal on adherent cultured human monocytes.

Figure 4. 5



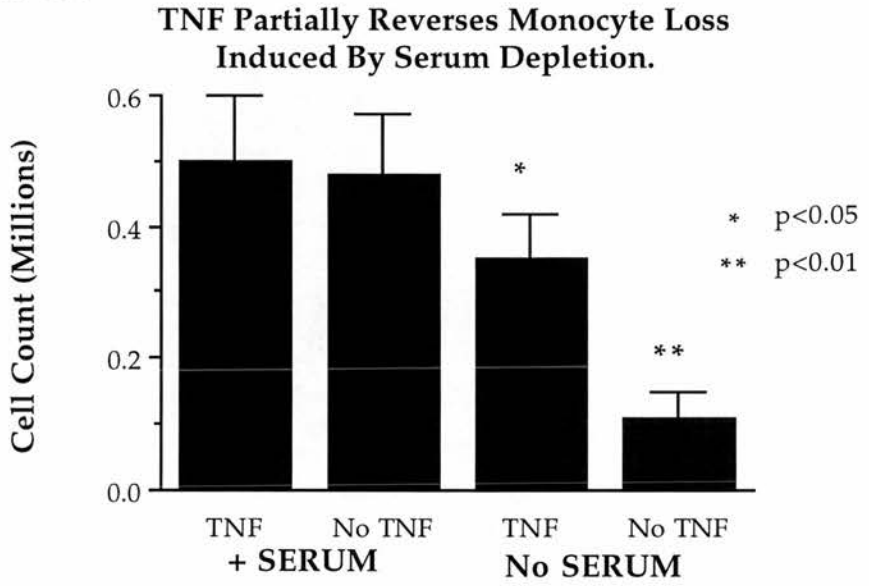
Legend for Figure 4. 5

Results are means ± SE for 4 paired experiments with human monocytes cultured in the presence or absence of serum with or without TNF-α 200 or 2000 units/ml, in 8 well slides under standard conditions for 24 hours. The percentage of apoptotic cells was determined morphologically by acridine orange fluorescent microscopy. Serum withdrawal significantly increased the percentage of apoptotic cells (p<0.001). There was no significant difference between percent apoptosis with or without 200 or 2000 units/ml TNF-

α in the presence of serum or in the absence of serum for 200 units/ml TNF-α. At 2000 units /ml TNF-α significantly reduced the percent apoptosis in the absence of serum $p<0.01$, $n = 4$.

The addition of TNF-α also significantly prevented the cell loss associated with suspension culture of monocytes in the absence of serum, shown in **Figure 4. 6** which demonstrates a significant but not complete protective effect of 2000 units/ml TNF-α in the absence of serum ($p<0.05$, $n = 4$). As with apoptosis counts, 200 units/ml TNF-α did not demonstrate any protective effect on cell recoveries (data not shown).

Figure 4. 6



Legend for Figure 4. 6

Effect of 2000 units/ml TNF-α on monocyte numbers after 5 days in suspension culture, seeded initially at 1×10^6/ml. Teflon foils stained after resuspension demonstrated no significant cell adherence. Results are the means ± SE of 4 separate experiments.

A similar result pertained for adherently cultured monocytes, counting the number of nuclei per high power field (HPF) after 5 days culture with or without serum and TNF-α. In these experiments, cell recovery in the absence of serum was 61% of that in the presence of serum. Addition of 2000 units/ml TNF-α increased this to 91% of the recovery in the presence of serum ($p<0.01$). Once again 200 units/ml TNF-α had no effect on this loss ($n = 4$).

There were no significant pro-apoptotic effects of serum withdrawal on Mø, with 2.4 ± 1.1% apoptosis in the absence of serum, the addition of up to 2000 units/ml TNF-α had no effect on this (2.0 ± 0.4% $n = 3$).

4. 2. 6. 3 TNF-α and Cycloheximide.

To further investigate the mechanism of TNF-α rescue of monocytes in the absence of serum I tested whether TNF-α could rescue cells from apoptosis induced by cycloheximide. Human monocyte derived Mø cultured on 8 well slides were exposed to cycloheximide (50 μM) for 24 hours when 13.6 ± 2.7% apoptosis was observed. Surprisingly the addition of 2000 units/ml TNF-α, followed by cycloheximide 15 minute later, doubled this level of apoptosis to 28.3 ± 1.1% ($p<0.05$). If the cycloheximide was added 15 minutes prior to TNF-α the level of apoptosis at, 26.6 ± 8.2%, was still almost double that observed with cycloheximide alone ($n = 4$). There was a highly significant decline in the number of nuclei per HPF when cycloheximide was added to the culture medium with 178 ± 9 nuclei per HPF in the control wells and 69 ± 8 in the cycloheximide treated wells ($p<0.001$, $n = 4$). The addition of both cycloheximide and TNF-α resulted in a further significant decrease in numbers to 44 ± 8 ($p<0.05$, $n = 4$).

4. 2. 6. 4 TNF-α with Other Cytokines.

Culturing monocytes or Mø in serum in the presence of the following four combinations: TNF-α with IL-1α, TNF-α with IL-4, TNF-α with IL-6 and TNF-α with all three (i.e. IL-1α, IL-4 and IL-6), all had no effect on the baseline levels of apoptosis. When monocytes were cultured in the absence of serum, the partial protective effect of TNF-α persisted in the presence these cytokine combinations (data not shown). These combinations of cytokines had a similar pro-apoptotic effect on Mø in the presence of cycloheximide to that of TNF-α alone, suggesting that the effect of TNF-α predominated both as a protective cytokine in the absence of serum and as a pro-apoptotic agent in the presence of cycloheximide.

4. 2. 6. 5 Effect of IL-1α, IL-4, IL-6, GM-CSF and TGF-β on Monocyte and Macrophage Apoptosis.

These cytokines had all been shown to influence apoptosis in myeloid cells. In previous studies, 1000 units/ml of IL-1α was found to retard apoptosis in monocytes induced by serum withdrawal (Mangan, 1991b). IL-4 (10 units/ml) prevents apoptosis in IL-3 dependant cells (Rodriguez-Tarduchy, 1992). IL-6 has been reported to induce apoptosis in U937 cells and PMN at 20 ng/ml (Afford, 1992) and at 1000 units/ml in the hematopoietic cell line, Y6 (Oritani, 1992). GMCSF at a concentration of 50 units/ml inhibits PMN apoptosis by 50% over 18 hours (Lee, 1993). TGF-β1 has been reported to induce myeloid cell apoptosis at 2 ng/ml (Lotem, 1992).

To investigate the relative effects of cytokines during differentiation, monocytes and Mø from the same donor were exposed to a range of concentrations of these cytokines in both suspension and adherent cultures and in certain experiments cells were also deprived of serum.

Table 4. 4

Effect of Cytokines on Monocyte and Macrophage Apoptosis.

Cytokine	Conc.	Monocytes (% Apop)		Mø (% Apop)	
	(units/ml)	No Serum	Serum	No Serum	Serum
Control	-	11.2 ± 2.8	2.0 ± 1.3	1.3 ± 0.3	0.9 ± 0.2
IL-1α	2250	9.4 ± 0.4	1.7 ± 0.6	1.0 ± 0.2	0.7 ± 0.3
IL-6	9000	8.8 ± 1.2	3.5 ± 0.8	1.3 ± 0.2	1.5 ± 0.5
GM-CSF	100	10.9 ± 0.9	3.8 ± 1.0	0.5 ± 0.3	0.6 ± 0.2
IL-4	1500	-	2.9 ± 0.9	-	0.5 ± 0.1
TGF-β	10 ng/ml	-	4.4 ± 0.4	-	1.3 ± 0.8
IL-1α + IL-4		-	2.9 ± 0.8	-	0.9 ± 0.2
IL-1α + IL-6		-	3.1 ± 0.9	-	0.3 ± 0.3
IL-4 + IL-6		-	2.2 ± 1.1	-	0.5 ± 0.1

Legend for Table 4. 4

Human peripheral blood monocytes were cultured adherent to 8 well slides in the presence or absence of 10% autologous serum (Controls) or with the addition of the cytokines stated at the concentrations stated (Conc.) in units/ml. The percentage of apoptotic cells was

determined morphologically by acridine orange staining at 42 hours. Data for cell recovery determined at the same times are shown in **Table 4. 4**. Monocytes from the same donors were matured *in vitro* in suspension for five days, these Mø were also plated onto slides and cultured in the presence or absence of serum and examined at 18 and 42 hours. Results are expressed as mean ± SE for 4 different donors.

As shown in **Tables 4. 4** and **4. 5** none of these cytokines had any effect on the levels of constitutive apoptosis for monocytes or Mø and did not influence cell recoveries over the time in culture. The percentage of apoptotic cells was determined at 18 and 42 hours but as the results were the same at 18 hours (except for monocytes cultured in the absence of serum), **Table 4. 4** only shows the results for 42 hours. For monocytes cultured in the absence of serum the mean percentage of apoptotic cells at 18 hours was 7.05 ± 1.3% compared with 10.8 ± 0.9% at 42 hours. As with the 42 hour results however, none of the cytokines tested had any effect on the percentage of apoptosis at 18 hours in the absence of serum. The addition of these cytokines also had no effect on the percentage of apoptosis for monocytes cultured in suspension (data not shown). Although only one concentration is shown for each cytokine in **Table 4. 4**, lower concentrations were used but did not affect the results; IL-1 was tested at 225 and 2250 units/ml, IL-4 at 150 and 1500 units/ml, IL-6 at 1800 and 9000 units/ml, GM-CSF at 10, 50 and 100 units/ml, TGF-β was only used at 10 ng/ml. Combinations were used at the highest concentrations only. IL-1α, IL-6 GM-CSF and TGF-β did not obviously influence cell morphology but using forward scatter parameters, monocytes cultured in the presence of 10 ng/ml TGF-β for 5 days were smaller than control cells: 43.8 ± 2.1 vs. 45.8 ± 1.9 ($p < 0.01$ n=3). IL-4 is discussed in section 4. 2. 7. 1.

Table 4. 5

Effect of Cytokines on Monocyte and Macrophage Recovery.

Cytokine	Conc.	Monocytes (as % of serum control)		Mø (as % of serum control)	
	(U/ml)	No Serum	Serum	No Serum	Serum
Control	-	76 ± 3.1	100 ± 2.1	100 ± 1.3	100 ± 1.7
IL-1α	2250	77 ± 3.6	102 ± 4.1	98 ± 3.4	97 ± 3.9
IL-6	9000	75 ± 3.3	107 ± 4.3	103 ± 4.5	96 ± 4.5
GM-CSF	100	82 ± 3.2	96 ± 1.9	99 ± 4.6	107 ± 4.3
IL-4	1500	-	96 ± 3.6	-	95 ± 5.1
TGF-β	10 ng/ml	-	102 ± 2.9	-	105 ± 3.9
IL-1α + IL-4		-	106 ± 4.0	-	96 ± 4.0
IL-1α + IL-6		-	108 ± 5.1	-	99 ± 5.3
IL-4 + IL-6		-	95 ± 4.9	-	96 ± 3.8

Legend for Table 4. 5

This table shows cell recoveries for cells cultured as described in **Table 4. 4**. Results are shown for adherent cultured monocytes and Mø and were determined by estimating the number of cells per high power

field (HPF), a minimum of 10 fields were examined per treatment. The number of cells per HPF for monocytes and Mø cultured in the presence of serum were taken as 100% and the cell recovery for cells cultured in the absence of serum or in the presence of cytokines were related to these values.

As noted in section 4. 2. 2, cell recoveries were lower in suspension than in adherent culture, none of the cytokines tested influenced this reduced recovery in suspension culture at 18 or 42 hours in any way (data not shown). Furthermore IL-1α, IL-6, GM-CSF and TGF-β did not affect the number of monocytes recovered after 5 days suspension culture, with a mean of 51 ± 7% recovery in control cultures and 53 ± 8, 49 ± 4, 50 ± 3, and 53 ± 7% in the presence IL-1α, IL-6, GM-CSF and TGF-β respectively (p = n/s, n = 4).

GM-CSF, IL-4, IL-6 and TGF-β, used at the same concentrations as in **Table 4. 4**, were also unable to protect Mø from cycloheximide induced apoptosis. Apoptosis counts in controls (10% autologous serum only) were 0.6 ± 0.2% after 14 hours, these compare with 12.2 ± 2% (n = 4) for cycloheximide treated cells and 12.6 ± 2.2%, 10.9 ± 1.9, 13.3 ± 0.9 and 13.1 ± 1.1 for GM-CSF, IL-4, IL-6 or TGF-β plus cycloheximide (50 μM) treated cells respectively, with equivalent recoveries for all treatments (p = n/s, n = 4) .

4. 2. 7. 1 Effect of IL-4 on Macrophage Morphology.

IL-4 induced many adherent cells to adopt a fibroblast-like morphology, with others having spindle shaped projections. IL-4 was the only cytokine to induce gross morphological changes, an effect which

predominated over other cytokines when cells were co-cultured with TNF-α, IL-1 or IL-6 and IL-4 together. These cells were more adherent even in suspension cultures in Teflon foils as shown in **Table 4. 6**.

Table 4. 6

Recombinant IL-4 Increases Monocyte Adherence to Teflon but does Not Alter Total Cell Recovery or Constitutive Apoptosis.

	Treatment					
	Control			IL-4		
Exp.	Count ($x10^6$/ml)		Apoptosis (%)	Count ($x10^6$/ml)		Apoptosis (%)
	Pre-EDTA	Total		Pre-EDTA	Total	
1	(0.65)	0.67	2.1	(0.44)	0.61	2.0
2	(0.49)	0.47	3.6	(0.38)	0.50	3.9
3	(0.35)	0.38	3.8	(0.20)	0.35	2.9
Mean	(0.49)	0.51	3.2	(0.34)	0.49	2.9

Legend for Table 4. 6

Monocytes, seeded at 1 x 10^6 /ml, were cultured in Teflon foils in the presence or absence of IL-4 (50 ng/ml) for 5 days when cells were resuspended and counted, the results are shown in brackets (Pre-EDTA). IL-4 induced an adherent morphology, the remaining adherent cells after resuspension were removed using EDTA (5 mM); the total of the pre and post EDTA counts are then shown (Total) demonstrating a much greater degree of adherence in the IL-4 treated cells. The percentage of apoptotic cells for the total cell population is shown and does not differ between treatments.

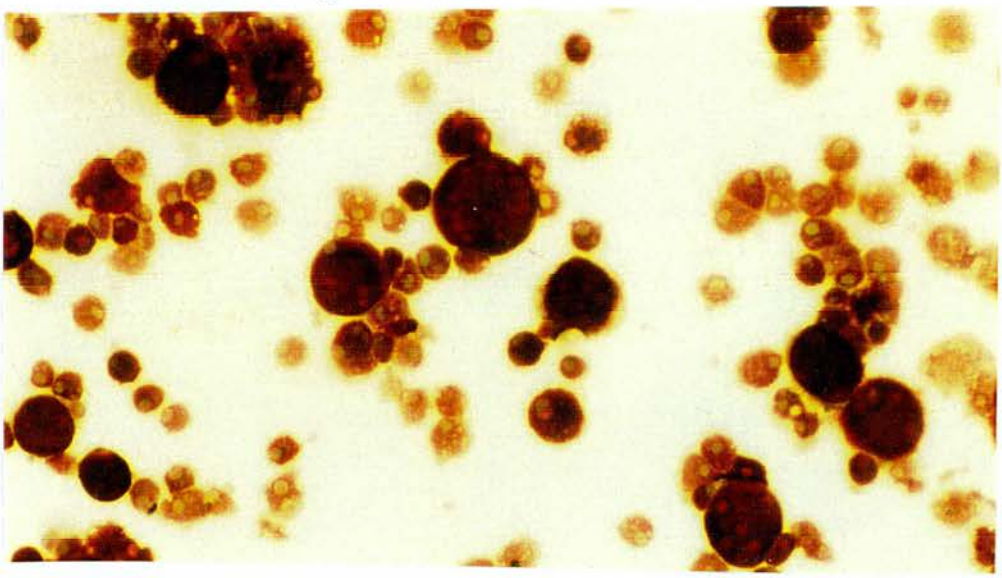
The number of monocytes after 42 hours adherent culture in the presence or absence of added IL-4 (50 ng/ml) were assessed. There were significantly more cells in the control than in IL-4 treated wells: 121 ± 7 cells per HPF in the control wells and 85 ± 7 in IL-4 wells ($p < 0.001$ $n = 6$). However more giant cells were present in the IL-4 treated wells and comparison of the number of nuclei per HPF eliminated any difference between treatments: 150 ± 7 vs. 168 ± 8 (p = n/s, n=6). Using forward scatter parameters on flow cytometry, IL-4 treated cells were also shown to be significantly larger than controls, (49.2 ± 1.5 vs. 45.8 ± 1.9 $p<0.05$ n=3).

4. 2. 7. 2 IL-4 Augments and Cycloheximide Prevents Giant Cell Formation.

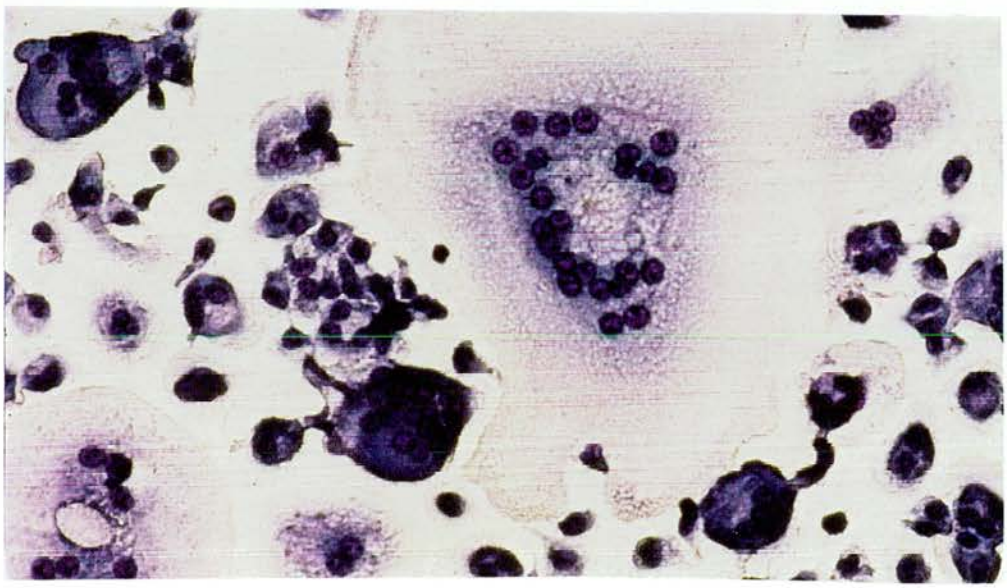
There was variability in the extent of giant cell formation between donors, certain donors were found to form large numbers of giant cells over the 5 days culture whilst others did not. Although donor variability was the predominant influence on giant cell formation, culture conditions could further alter the number and multinuclearity of giant cells. Suspension culture of monocytes significantly reduced the formation of cells with more that two nuclei when compared with adherent culture for five days (4.2 ± 0.7% vs. 11.2 ± 1.5%, $n = 7$ respectively). There was no difference when the percentage giant cells was determined from Dif Quik™ stained cytospin preparations or by acridine orange fluorescent microscopy of resuspended cells. TNF-α had no effect on giant cell formation for any donors, IL-4 significantly increased giant cell formation and cycloheximide prevented multinucleate giant cell formation as shown in **Figure 4. 7**.

Figure 4. 8 Giant Cell Formation in Macrophage Cultures.

i) Giant Cells in 5 Days Adherent Human Macrophage Culture, Stained for Non-Specific Esterase.



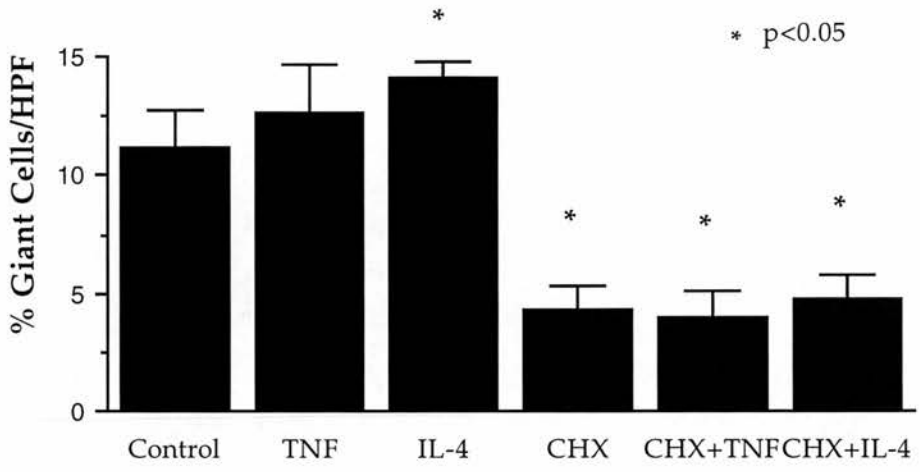
ii) IL-4 Treatment Increases the Number of Nuclei in Giant Cells.



Legend for Figure 4. 8 Two photomicrographs of adherent cultured human Mø demonstrating different giant cell morphologies. Figure **i**) shows typical giant cell formation from a donor whose Mø regularly formed a large numbers of giant cells in culture (x 200). Figure **ii**) shows the massive size of some giant cells after exposure of monocytes to IL-4 (1500 units/ml) for 5 days (section 4. 2. 7. 1) (x 200).

Figure 4. 7

IL-4 Promotes and Cycloheximide inhibits Giant Cell Formation



Legend for Figure 4. 7

Monocytes were cultured on 8 well slides for 5 days in the presence of 10% autologous serum (Control), TNF-α (2000 units/ml), IL-4 (1500 units/ml), cycloheximide (CHX; 50 μM), or both cycloheximide and TNF-α (CHX + TNF) or cycloheximide and IL-4 (CHX + IL-4). TNF-α and IL-4 were added at the start of culture but CHX was added on day 4. Giant cells determined by Dif Quik™ staining adherent cells and counting % giant cells. Results are means ± SE , n = 7.

IL-4 (1500 units/ml) had variable effects when a high level (12% or more) of giant cell formation was apparent, increasing fusion in one donor but with no effect in two others. Where giant cell formation was less than 6%, IL-4 significantly up-regulated giant cell formation in adherent cultures, from 5.1 ± 1.4% in control cultures to 9.8 ± 0.9% in IL-4 treated cultures (1500 units IL-4/ml), ($p<0.05$, n = 4). This effect was dose dependant with lower doses (e.g. 150 units/ml) being less effective. The more dramatic effect of IL-4 (1500 units/ml) was that it increased the number of nuclei per giant cell, control cultures having a mean of 4.6 ±

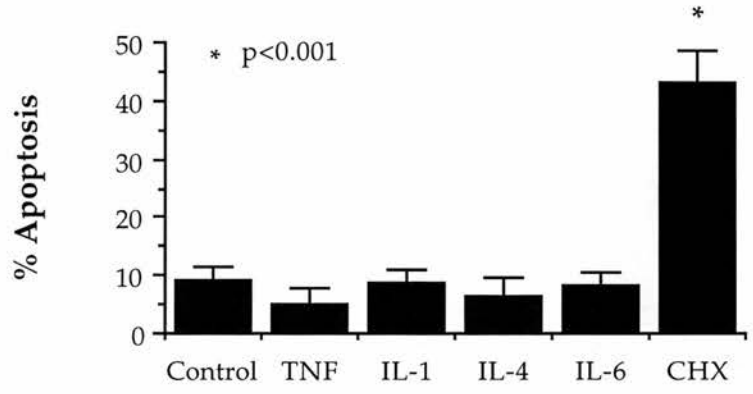
0.6 nuclei per giant cell whilst IL-4 treated cells had 11.4 ± 2.2 nuclei per giant cell ($p<0.01$, $n = 7$) (**Figure 4. 8**). This effect on the number of nuclei per giant cell was also seen with low dose IL-4 (150 units/ml) where multinucleate giant cells had a mean of 10.8 ± 2.0 nuclei/cell ($p<0.01$, $n = 3$). Other cytokines tested {GM-CSF (50 units/ml) IL-1α (1000 units/ml), IL-6 (9000 units/ml) and TGF-β1 (10 ng/ml)} had no effect on giant cell formation. When cycloheximide (50 μM) was added on day 4 of culture there was a significant decrease in the percentage giant cells present at day 5, with a mean of 4.8 ± 0.8% giant cells after addition of cycloheximide compared with 11.2 ± 1.5% in control cultures ($p <0.05$, $n = 7$). There were 5.1 ± 1.4% giant cells in adherent culture monocytes after 4 days in culture suggesting that giant cell formation may be a process dependant upon new protein production.

4. 2. 8 Cytokines Do Not Affect Alveolar Macrophage Apoptosis.

The baseline level of apoptosis in human alveolar Mø suspension cultures immediately after washing and resuspension was 8.8 ± 1.8% ($n = 8$) as determined by acridine orange fluorescence microscopy, this remained unchanged after 18 hours in culture (9.0 ± 2.5%, $n = 8$). **Figure 4. 9** shows the effect of a range of cytokines on alveolar Mø apoptosis and compares this with the effect of cycloheximide. Serum withdrawal for 18 hours did not alter alveolar Mø apoptosis, (8.8 ± 2.3%, $n = 4$) and TNF-α had no additional effect on the levels of alveolar Mø apoptosis in the absence of serum (7.1 ± 0.9%, $n = 3$), no assessment was made at 84 hours due to insufficient cell numbers.

Figure 4. 9

Cytokines Exert Little Influence on Alveolar Mø Apoptosis



Legend for Figure 4. 9

Alveolar Mø, cultured in standard conditions in Teflon foils in the presence of 10% human AB serum were incubated with TNF-α (2000 units/ml), IL-1α (1000 units/ml), IL-4 (50 ng/ml), IL-6 (9000 units/ml), cycloheximide (CHX; 50 μM) or no addition (Control) for 18 hours. The percent apoptosis was determined morphologically and results expressed as mean ± SE for 4 different donors.

4. 2. 9 Effect of Cytokines on Murine Bone Marrow Macrophage Cultures.

4. 2. 9. 1 Effect of TNF-α, IL-1, IL-4, IL-6 and TGF-β on Murine Bone Marrow Derived Macrophages.

Murine bone marrow Mø, cultured as described in section 2. 5. 4, were used on day 7. As shown in **Table 4. 7** the addition of the following cytokines; IL-1, IL-4, IL-6 or TGF-β1 had no effect on apoptosis or on cell recovery. Apart from IL-4, all these cytokines cross reacted with murine cells, for these experiments on murine Mø, recombinant murine IL-4 was purchased.

Table 4. 7

Effects of Cytokines on Murine Bone Marrow Macrophage Survival.

Treatment	24 Hours		72 Hours	
	Apoptosis (%)	Cell Counts (x 10^6/ml)	Apoptosis (%)	Cell Counts (x 10^6/ml)
Control	0.3	1.8 ± 0.2	2.0	1.40 ± 0.3
TNF-α 200 units	0.25	1.94 ± 0.3	0.6	1.90 ± 0.4
TNF-α 2000 units	0.05	1.70 ± 0.3	0.0	2.20 ± 0.2*
TNF-α 2000 units+ CHX	30.5*	1.58 ±0.3	100*	0.6 ± 0.1 §
CHX alone	17.2*	1.54 ± 0.3	100*	0.72 ± 0.2 §
IL-1	0.24	1.85 ± 0.1	3.0	1.39 ± 0.2
IL-4	1.4	1.90 ± 0.4	1.9	1.38 ± 0.3
IL-6	1.25	1.88 ± 0.4	2.8	1.37 ± 0.2
TGF-β	1.75	1.64 ±0.3	3.0	1.60 ± 0.3

* significantly greater than control $p<0.05$

§ significantly lower than control $p<0.05$

Legend for Table 4. 7

Effect of various cytokines on percentage apoptosis and cell recovery of murine bone marrow derived Mø in suspension culture. Cells were seeded at 0.375×10^6/ml, washed on day 5 and resuspended at 1×10^6/ml. On day seven, 1.75% of cells were apoptotic and cell counts were $1.75 \pm 0.3 \times 10^6$/ml. Cells were divided into 9 Teflon foils and treatments added as per Table; control cells were cultured in medium plus 25% FCS and 25% L929 cell supernatant. Cell recovery was expressed as mean ± SE, n = 3. Percentage apoptosis did not vary by more than 10% of the mean for each treatment hence only the means are shown.

There was no difference in the cell recovery after 24 hours for any of the treatments, but by 72 hours significantly fewer cycloheximide treated cells were recovered ($p<0.05$, n = 3) and significantly more TNF-α (2000 units/ml) treated cells were recovered ($p<0.05$, n = 3). As with human Mø, cycloheximide promoted apoptosis and this effect was augmented over 24 hours by TNF-α. DNA electrophoresis also demonstrated greater fragmentation with the combination of cycloheximide and TNF-α than for cycloheximide treated cells alone (**Figure 4. 10**).

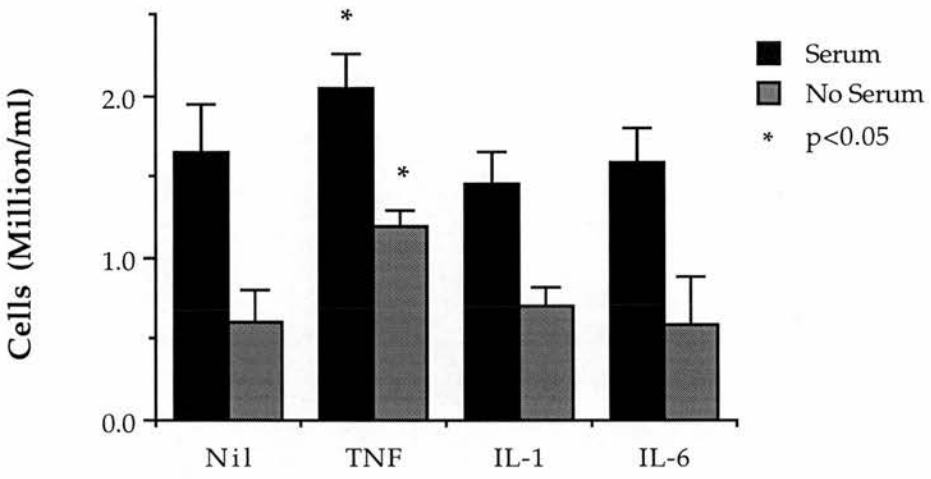
4. 2. 9. 2 Murine Bone Marrow Derived Macrophages: Growth Factor Depletion and Effect of TNF-α, IL-1α and IL-6.

As discussed in section 4. 2. 4, culturing murine bone marrow derived Mø for 42 hours in the absence of serum and CSF-1 significantly reduces

cell recovery. **Figure 4. 11** shows that TNF-α but not IL-1 or IL-6 significantly augments cell recovery both in the presence and absence of serum plus CSF-1.

Figure 4. 11

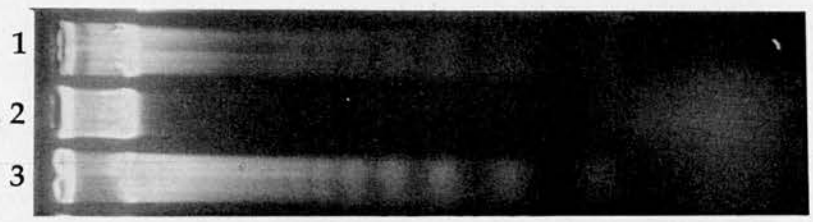
TNF Increases Murine Bone Marrow Mø Survival



Legend for Figure 4. 11

Cells were seeded in Teflon foils at 0.375 x 10^6 cells/ml, washed and resuspended at 1 x 10^6/ml on day 5. On day 7 cells were washed twice and resuspended at 1.75 ± 0.3 x 10^6/ml in either medium alone (No Serum) or medium + FCS and L929 supernatant (Serum) in the presence of TNF-α (2000 units/ml), IL-1α (1000 units/ml), IL-6 (9000 units) or and equal volume of culture medium (Nil). Cell recovery after 42 hours, expressed as mean ± SE for 3 experiments. Cell recovery in the absence of serum with Nil, TNF-α, IL-1α or IL-6 were all significantly lower than that for the corresponding cells cultured in the presence of FCS + CSF-1 ($p<0.05$, n = 3). TNF significantly increased the cell recovery from that of Nil or IL-1 or IL-6 in the presence and absence of serum plus CSF-1.

Figure 4. 10 TNF-α Augments Cycloheximide Induced Macrophage Apoptosis.



Legend for Figure 4. 10 Photomicrograph of ethidium bromide stained DNA gel taken under ultraviolet light. Murine bone marrow derived Mø were cultured for 7 days then exposed to cycloheximide (50 μM) (Lane **1**), an equal volume of the diluent - ethanol (Lane **2**) or cycloheximide (50 μM) plus TNF-α (2000 units/ml) (Lane **3**) for 24 hours. One million cells per well were lysed and their DNA extracted. The agarose gel demonstrates the ladder pattern typical of apoptosis in the cycloheximide treated cells but not the control (ethanol treated) cells. TNF-α significantly increased the percentage of apoptotic cells (section 4. 2. 9. 1), this was also clearly seen on the gel.

4. 2. 10 Effect of Dexamethasone and cAMP on Macrophage Apoptosis.

4. 2. 10. 1 Dexamethasone.

Glucocorticoids are known to promote apoptosis in thymocytes (Wyllie, 1980) and eosinophils (Stern, 1992) although interestingly they delay apoptosis in PMN (Meagher, 1996). In view of the perceived anti-inflammatory action and pro-apoptotic effect of dexamethasone on various cell types, its effect on Mø survival was investigated. The standard concentration used to promote apoptosis on thymocytes and eosinophils is 1μM, **Table 4. 8** shows that dexamethasone over a range of concentrations from 0.5 to 2.0 μM had no effect on Mø apoptosis. Furthermore dexamethasone did not prevent or enhance the pro-apoptotic effect of cycloheximide. As with Mø, dexamethasone had no effect on human monocyte apoptosis, there being 5.9 ± 1.2 % apoptotic cells after 18 hours suspension culture in the presence of 10% autologous serum alone and 4.8 ± 2.0% with the addition of 1 μM dexamethasone. Dexamethasone was unable to prevent the pro-apoptotic effect of cycloheximide, with 29 ± 4.9% apoptotic monocytes after 18 hours incubation with 50 μM cycloheximide and 26.4 ± 3.8% with the combination of 50 μM cycloheximide and 1 μM dexamethasone (n = 3). Dexamethasone also had no effect on the percent apoptosis after 18 hours when either monocytes, monocyte derived Mø, human alveolar Mø or murine bone marrow derived Mø were cultured in the absence of autologous serum (data not shown).

Table 4. 8

Dexamethasone does Not Promote Macrophage Apoptosis or Prevent Cycloheximide Induction of Apoptosis.

Treatment	Apoptosis (%)	Cell Count (x 10^6/ml)	Non-viable (%)	p
Control	2.6 ± 1.2	0.64 ± 0.07	3.7 ± 0.8	
Dexamethasone				
0.5 μM	3.1 ± 0.8	0.60 ± 0.06	3.8 ± 0.5	n/s
1.0 μM	2.6 ± 0.9	0.63 ± 0.09	4.3 ± 0.9	n/s
2.0 μM	4.2 ± 1.4	0.61 ± 0.13	3.1 ± 1.4	n/s
CHX	16.0 ± 1.5	0.57 ± 0.05	10.4 ± 1.1	0.04
Dex + CHX	15.2 ± 0.7	0.59 ± 0.07	11.6 ± 1.4	0.01

Legend for Table 4. 8

Human monocyte derived Mø were cultured in Teflon foils at 0.60 x 10^6/ml in the presence of 10% autologous serum (Control) or treated with dexamethasone (0.5 to 2.0 μM), cycloheximide (CHX; 50 μM) or a combination of both dexamethasone 1.0 μM and cycloheximide (Dex + CHX). After 18 hours cells were resuspended, counted, viability determined and the percentage apoptosis counted morphologically by acridine orange fluorescent microscopy. The percent apoptotic cells in the control foils was compared with those for other treatments, the significant p values are shown. Results are the means ± SE for 4 separate experiments.

4. 2. 10. 2 db-cAMP.

In view of the postulated involvement of cAMP in the transduction of the apoptotic signal (McConkey, 1990), I investigated the effect of cAMP on Mø apoptosis, adding the membrane permeable analogue db-cAMP to increase intracellular cAMP levels. This had a delayed effect, with no significant difference in percentage of apoptotic Mø in control or db-cAMP cultures after 24 hours, however by 72 hours db-cAMP significantly increased the percentage of apoptotic Mø, as shown in **Table 4. 9**.

Table 4. 9

Dibuteryl-cyclic Adenosine Monophosphate has a Late Pro-apoptotic Action on Human Macrophages.

Treatment	Time (Hours)	Apoptosis (%)	Cell Number ($x10^6$/ml)	Non-viable (%)
Control	24	2.4 ± 0.7	0.95 ± 0.09	2.1 ± 0.9
	72	1.9 ± 0.7	0.82 ± 0.07	2.9 ± 1.1
db-cAMP	24	5.3 ± 1.6	0.89 ± 0.08	4.9 ± 1.2
	72	14.3 ± 2.4*	0.71 ± 0.10	19.1 ± 4.2

Legend for Table 4. 9

Human monocyte derived Mø were cultured in Teflon foils at 1.0 x 10^6/ml in the presence of 10% autologous serum (Control) or treated with 2 mM db-cAMP. After 24 and 72 hours cells were resuspended, counted, viabilities determined and the percentage apoptotic cells counted morphologically. There were no significant differences between Control and db-cAMP treated cells after 24 hours, however

by 72 hours, there were significantly (*) more apoptotic cells in the db-cAMP treated foils ($p < 0.05$, $n = 4$).

A similar late pro-apoptotic effect was found on monocytes cultured adherent to 8 well slides (data not shown) and on murine bone marrow derived Mø, incubation of 7 day cells with db-cAMP for 72 hours increased the percentage of apoptotic cells from 3.8 ± 0.6 to 12.2 ± 0.5 ($p < 0.05$, $n = 3$). As with other culture systems that induced monocyte apoptosis, "ghost cell" bodies were observed in the presence of db-cAMP associated monocyte apoptosis.

4. 3 DISCUSSION.

Monocyte survival during *in vitro* culture is affected by the presence of serum and by adherence to culture plastic. In contrast to culture in the presence of serum, serum withdrawal leads to a 25% loss in numbers of adherent monocytes as seen in **Figure 4. 3**. Suspension culture in the presence of serum leads to a 43% loss of monocyte number, which increased to a 76% loss when cultured both non-adherently and in the absence of serum. These results are comparable with previously published data, demonstrating a significant loss of human monocytes in culture that is increased by serum withdrawal and further increased by suspension culture (Johnson, 1977; Andreesen, 1983; Becker, 1987; Helinski, 1988; Brugger, 1991; Mangan, 1991a and b). These results also confirm the findings of Andreesen *et al.* and Helinski *et al.* that despite losses, monocytes can be matured successfully to Mø in Teflon foils (Andreesen, 1983; Helinski, 1988). Monocyte losses are accompanied by morphological and biochemical evidence of apoptosis but without evidence of necrosis. The percentage of apoptotic monocytes recorded at

each time point (**Figure 4. 4**) were consistently lower than cell losses (**Figure 4. 3**) and "ghost cell" bodies were commonly observed at later time points when monocytes were cultured in the absence of serum, in the presence of cycloheximide or with the combination of cycloheximide and TNF-α suggesting that they represent "late" apoptotic monocytes, as discussed in Chapter 3.

One key finding in this work is that there are powerful survival differences between the monocyte and the Mø. Monocyte losses in suspension are in the order of 22% over 42 hours even in the presence of serum whilst over the same time course Mø losses are approximately 8%. Furthermore monocyte losses are augmented by serum withdrawal, whereas Mø are resistant to these effects. Although several papers describe methods for long term Mø culture (Helinski, 1988; Bennett, 1993), no comparison of the rates on monocyte and Mø death had been made and this distinction between monocyte and Mø survival had not been investigated before although very recently Munn *et al.* also documented this differential sensitivity to apoptosis between monocytes and Mø (Munn, 1995). This raises the important question of the mechanism of this increased resistance of the Mø to the induction of apoptosis which we address in subsequent chapters.

Adult mouse serum contains approximately 600 U/ml of M-CSF, which is adequate for both cell survival, although proliferation may require more (Tushinski, 1982). Similarly the reported normal level of M-CSF in human serum is 550 ± 110 U/ml, hence withdrawal of this factor alone may have had a significant effect on cell survival (Brugger, 1991). Becker *et al.* showed that addition of 1000 U/ml of M-CSF to human monocyte

cultures provided better cell recovery than 5% serum (Becker 1987), whilst Mangan *et al.* showed that GM-CSF, M-CSF, IFNγ and IL-1 all prevented apoptotic cell death, and found that IL-1 was the most potent (Mangan, 1991b). There are conflicting reports in the literature as to whether colony-stimulating factors can induce synthesis of pro-inflammatory cytokines and thus provide an additive protective effect (Hamilton, 1993a and b). Data presented in this thesis demonstrates that serum significantly promoted monocyte survival, while both serum and CSF-1 promoted murine bone marrow derived Mø survival. TNF-α exerted a significant survival effect on monocytes and murine bone marrow derived Mø but the colony stimulating factor GM-CSF and the cytokines IL-1α, IL-4, IL-6 and TGF-β all were shown to have no influence on monocyte or Mø apoptosis or survival.

Thus cytokines which are predominant during the early inflammatory response (IL-1, GM-CSF and IL-6) as well as those with defined role during late inflammation and resolution (IL-4, GM-CSF and TGF-β1) had remarkably little effect on monocyte and Mø survival. All cytokines were added in concentrations known to exert pro/anti-apoptotic effects on other cells. Monocytes cultured in the presence of autologous serum die steadily by apoptosis and none of the cytokines tested altered this in any way, despite evidence for a pro-apoptotic effect of IL-6 (Afford, 1992; Oritani, 1992) and TGF-β1 (Lotem, 1992) and an anti-apoptotic effect of IL-1 (Mangan, 1991b), IL-4 (Rodriguez-Tarduchy, 1992) and GM-CSF (Lee, 1993) on other myeloid cells. The lack of cytokine effect was irrespective of the duration of treatment - cells were examined after 6, 18, and 42 hours and 5 days - and irrespective of whether the cytokines were only added at the beginning or if they were continually supplemented

throughout the culture period. As monocyte survival is greater when adherent, cytokines were added to both suspension and adherent monocyte cultures to see if this could unmask an effect on cell survival but neither cell recovery nor apoptosis were influenced by any of the cytokines tested for either of these culture conditions. The level of monocyte apoptosis was found to be increased by serum withdrawal and in this study TNF-α was shown to inhibit this effect.

Mø were resistant to the induction of apoptosis, consistent with their recognized long life *in vivo* (van Furth 1992). None of the cytokines tested, either on their own or in combination, were shown to promote apoptosis in Mø cultures, a finding which was consistent among those sources of Mø available to us: human peripheral blood monocyte derived Mø, human alveolar Mø and murine bone marrow derived Mø.

The only cytokine which had any significant effect on apoptosis was TNF-α. This could be shown to significantly but not completely prevent the pro-apoptotic effect of serum withdrawal (**Figure 4. 5**) on human monocytes and to promote survival (**Figure 4. 6**). TNF-α also partially inhibited the pro-apoptotic effect of growth factor (CSF-1) withdrawal on murine bone marrow derived Mø (**Figure 4. 11**). In addition TNF-α promoted murine bone marrow Mø proliferation in the presence of serum and CSF-1 (**Table 4. 7**). As there was no pro-apoptotic effect of serum withdrawal on Mø, no survival effect of TNF-α could be demonstrated for these cells. The effect of TNF-α was dose dependent, 2000 units/ml were protective whilst 200 units/ml were not.

When TNF-α was combined with cycloheximide it had a powerful pro-apoptotic effect, significantly augmenting cycloheximide induced apoptosis in both human and murine Mø. One consequence of this effect was a reduction in total cell recovery when TNF-α and cycloheximide were combined. Synergism between TNF-α and cycloheximide has been reported for PMN, with more than a fivefold increase in apoptosis (Tsuchida, 1995). Furthermore, although PMN and monocytes required similar concentrations of cycloheximide alone to induce apoptosis (36 vs. 50 μg/ml respectively), when combined with TNF-α, PMN required much lower concentrations of cycloheximide than monocytes to induce similar levels of apoptosis (3.6 vs. 50 μg/ml) (Takeda, 1993; Tsuchida, 1995).

TNF-α has been shown to cause apoptosis in a number of primary cell types including PMN (Takeda, 1993; Tsuchida, 1995), thymocytes (Hernandez-Castelles, 1993) and endothelial cells (Polunovsky, 1994). The pro-apoptotic effect has also been well documented in a range of cell lines including U937 (Jarvis, 1994), HL60 (Obeid, 1993), KYM-1 (a rhabdomyosarcoma cell line; Grell, 1994) and the T cell line PC60 when transfected with TNFR (Vandenabeele, 1995). The inability of TNF-α alone to induce apoptosis in monocytes and Mø is thus of interest, especially as these cells express functional TNF receptors (section 5. 2. 1).

One possible explanation for the results obtained with TNF-α is that monocytes and murine bone marrow Mø are dependent for their survival upon anti-apoptotic factor(s) which are induced by growth factor (or serum) stimulation via a protein synthesis dependent mechanism. In the absence of growth factors (or serum) TNF-α can continue to induce production of this factor but when protein synthesis is inhibited by

cycloheximide this action is blocked and other pro-apoptotic effects of TNF-α are unmasked, contributing to increased cell death. The normal serum levels of TNF-α in non-septic subjects are very low suggesting that TNF itself does not account for this serum effect. It is attractive to speculate that the redox state of the cell may be important in this latter process. It has been reported that transiently inducible protective proteins such as superoxide dismutase and heat shock proteins render cells resistant to TNF-α-induced cytotoxicity (Wong, W. 1988) and could partially compensate for the loss of anti-oxidant protection normally afforded by serum. TNF-α also induces increased superoxide generation and reactive nitrogen intermediates (Hauschildt, 1992; Hennet, 1993). Hence TNF-α stimulation would induce an increase in ROI but their pro-apoptotic effect would be balanced by co-induction of superoxide dismutase and heat shock proteins. Cycloheximide would prevent the synthesis of these protective proteins unmasking TNF-α induced free radical toxicity.

Data presented in this thesis demonstrates that serum depletion promotes monocyte apoptosis, an effect which can be partially inhibited by TNF but not by IL-1α, IL-4, IL-6, GM-CSF or by TGF-β1. The protective effect that TNF-α accords monocytes during serum withdrawal has been reported by Mangan *et al.* who showed that a range of cytokines and inflammatory agents could delay monocyte apoptosis (Mangan, 1991a and b). Monocyte survival was promoted by IL-1β, TNF-α, LPS, GM-CSF, IL-1-α, IFN-γ, M-CSF and IL-3 in descending order of effectiveness, whilst IL-6, TGF-β1, MCP-1, fMLP and C5a all had no effect. There are a number of differences between data reported by Mangan *et al.* and that presented here.

Already mentioned is the failure of GM-CSF and IL-1α to protect monocytes from serum depletion induced apoptosis. Furthermore, even within the protection afforded by TNF-α there are some differences. I demonstrated little effect with 200 units/ml TNF-α and only a 50% protection with 2000 units/ml TNF-α whilst Mangan *et al.* using 1000 units/ml TNF-α obtained over 75% protection against monocyte apoptosis (Mangan, 1991a). Several groups have demonstrated that rather than protecting Mø from apoptosis, IL-1α and IL-1β (but not TNF-α or IL-6) are processed and released during Mø apoptosis, suggesting that IL-1 may in fact be signalling terminal cellular injury (Hogquist, 1991a; Sarih, 1993b; Zychlinsky, 1994). Furthermore Brugger *et al.* have shown that in a serum free system; IFN-γ, IL-1α or β, IL-4 or IL-6 had no effect on monocyte survival (Brugger, 1991). Interestingly, Moore *et al.* have recently demonstrated that TNF-α was able to prevent apoptosis induced by removal of M-CSF in murine bone marrow derived Mø but that TGF-β, IL-1 and IL-6 had no effect (Moore, 1994), however they also demonstrated that GM-CSF could prevent apoptosis induced by M-CSF withdrawal. Together these data indicate that a clear role for TNF-α exists in protecting monocytes from the pro-apoptotic effects of serum withdrawal, while the role of other cytokines remains uncertain.

There are several possible explanations for the different findings of this work and that of Mangan *et al.*. Importantly the methods of monocyte isolation used were different, in that Mangan *et al.* employed CCE whereas my study relied on monocytes prepared by density gradient and adherence separation (Mangan, 1991a and b). Although final monocyte purity is very similar, these two methods could be selecting different populations. As I demonstrate in section 3. 2. 1a, a significant proportion

of monocytes do not remain adherent to plasma coated plates and are thus lost during preparation, this population is retained by CCE and may exhibit different survival characteristics. Certainly adherence reduces Mø sensitivity to the induction of apoptosis but whether this effect persists after cells have been detached is not known (Khan, 1993). Adherence may also "prime" monocytes such that later stimulation results in a significant increase in IL-1 production (Schindler, 1990). Despite these differences between my experiments and those of Mangan *et al.* the cell recoveries after 42 hours in suspension culture in the absence of serum are not too dissimilar. Comparison of CCE and adherence derived monocyte phenotype does not demonstrate any significant differences in cell-surface molecule expression (Dransfield, 1988) thus differences may be more subtle and related to maturation or activation.

The murine bone marrow derived Mø system described here generally parallels the human system and none of the cytokines tested promoted apoptosis. As with monocytes, TNF-α prevented apoptosis induced by growth factor depletion and in this murine system TNF-α also increased cell recovery. It is interesting to compare this with the results of Witsell *et al.* who demonstrated that TNF-α acts as a monocyte differentiation factor decreasing murine bone marrow proliferation (Witsell, 1992). This effect was only evident over a narrow "window" of time, on day 3 of *in vitro* differentiation, when TNF-α acted to switch monocyte colony forming units from proliferation to Mø differentiation. The survival effect I have shown for TNF-α was evident on day 7 to 10 of *in vitro* differentiation, outside the window shown by Witsell and at a time point they did not examine. By this time monocytes have differentiated into functional Mø, are competent phagocytes and express typical Mø surface

markers. Without TNF-α cell numbers decline from day 7 to 10 despite the presence of serum and CSF-1. In the presence of TNF-α the cell recovery on day 10 is only slightly greater than that on day 7 but significantly greater than day 10 cell recoveries in the absence of TNF-α (**Table 4. 7**), suggesting that the TNF-α effect may be to promote survival rather than proliferation. The relative contributions of cell proliferation and prolonged cell survival to this results would need to be assessed to address this issue. Again, as with human monocytes, additive effects between cycloheximide and TNF-α are demonstrated for both apoptosis and cell recovery.

The level of apoptosis in these different cell cultures may be related to their proliferation rates. After six hours culture in the absence of serum the percentage of apoptotic cells are: 20% for U937 cells - highly proliferative cells (Tushinski, 1982); 14% for murine bone marrow derived Mø - a recently proliferating system; 8% for human monocytes - cells capable of limited proliferation (Cheung, 1992; van Furth, 1992, Bennett, 1993); 2% for alveolar Mø and 0.85% for human monocyte derived Mø - non-proliferative or with a very low proliferative rate (van Furth, 1992). This suggestion is of interest in that many genes involved in the apoptotic pathway also act as regulators of cell division. The possible contributions of various oncogenes involved in the apoptotic process is considered further in Chapter 6.

There is a steady and significant decline in murine bone marrow derived Mø numbers over 42 hours when depleted of serum and CSF-1, with a 30% loss by 18 hours and 66% loss by 42 hours. In comparison to this increased cell loss, the percentage of apoptotic cells in culture decreases

between 18 hours (38% apoptotic cells in the absence of serum and CSF-1) and 42 hours (5% apoptotic cells). Repeated morphological examinations confirm the absence of significant necrosis and DNA gel electrophoresis confirm continued apoptosis throughout this time period. This decline in cell numbers indicates either increasing fragility and reduced life-span of the apoptotic cells during the process of apoptosis induced by serum withdrawal, or rapid clearance of the apoptotic cells by functional Mø. As these cells were cultured in suspension, no "ghost cell" bodies were seen, the act of resuspension destroying them (section 3. 2. 3). Although phagocytosed apoptotic cells could be seen occasionally within the functional Mø this was not quantifiable, so no distinction can be drawn at present as to the relative contribution of these two processes in the clearance of apoptotic Mø *in vitro*. In the light of the efficient clearance of apoptotic PMN by phagocytic Mø (Savill, 1989a), it is likely that phagocytosis would be the relevant clearance mechanism *in vivo*.

The data for U937 cells demonstrates a requirement for serum for continued growth and in the absence of serum they cease to multiply and begin to die by apoptosis. In RPMI medium alone this death occurred rapidly (30 minutes) whereas in IDMEM alone it was slightly delayed (6 hours), thus an extra factor beyond that of serum withdrawal was involved. Differences in the buffering capacity between the media may however be a relevant factor as noted in section 4. 2. 1.

Alveolar Mø are long lived cells and more akin to peripheral blood matured Mø than to monocytes. They also die by apoptosis constitutively in culture and serum withdrawal acts to promote this. As noted in section 4. 2. 5, they are not acutely sensitive to serum depletion with no

significant apoptosis detectable at 18 hours and even in the absence of serum 70% of cells are still recoverable after 84 hours in culture. Thus, as with monocyte derived Mø, alveolar Mø are shown to be more resistant to apoptosis than the monocytes.

I wanted to determine the significance of fates other than apoptosis for monocytes. There was little evidence that necrosis represented a major fate for monocytes or Mø and none of the agents investigated induced this *in vitro*. Fusion into multinucleate giant cells was observed during *in vitro* culture. I showed that suspension culture and cycloheximide treatment both inhibited giant cell formation and that IL-4 had a small positive dose dependant effect especially in those donors with a low intrinsic level of giant cell formation. IL-4 was also shown to significantly increase the number of nuclei per giant cell. Donor heterogeneity however was the greatest influence on giant cell formation. One possibility is that differences in the levels of IL-4 of autologous serum may account for differences in the tendency to form giant cells. This could be investigated using antibody inhibition studies and by determining serum IL-4 levels. A positive effect of IL-4 on giant cell formation has been reported on murine bone marrow Mø and alveolar Mø (McInnes, 1988). Suspension culture has also been shown to decrease cell fusion, confirming my findings (Enelow, 1992). Enelow *et al.* also showed that TNF-α and IL-6 alone had no effect on giant cell formation which is in accord with my results (Enelow, 1992). Certainly the usual fate of the Mø does not seem to be that of giant cell formation, rather this is a specific morphology adopted in response to particular circumstances such as in granulomas. IL-4 also caused a gross change in the morphology of Mø. This increase in size and altered morphology has

been reported by te Velde *et al.* who showed that as little as 50 units/ml IL-4 caused morphological change in monocytes by the 5th day in culture and an increase in antigen presenting function (te Velde, 1988).

The glucocorticoid dexamethasone had no effect on monocyte or Mø apoptosis, neither did it alter the extent to which cycloheximide induced Mø apoptosis. Glucocorticoids are powerful inducers of apoptosis in thymocytes (Wyllie, 1980), hence this lack of effect on Mø again underlines differential controls on Mø and lymphoid cell survival. This differential action of glucocorticoids however appears cell rather than lineage specific, as emphasized by differences among granulocytes where glucocorticoids delay the onset of PMN apoptosis and promote eosinophil apoptosis (Meagher 1996).

cAMP, an activator of protein kinase A, was shown to exert a pro-apoptotic effect on Mø but the magnitude of this was small and of late onset, with only 10% apoptosis after 72 hours, thus it is possible that this is the result of an indirect action. This pro-apoptotic effect, although small, is of interest as cAMP exerts the opposite effect on PMN (Rossi, 1995). Exposure of U937 cells to db-cAMP however leads to a reduction in their size with associated nuclear condensation (Sheth, 1988) suggesting that like Mø, elevations of cAMP may induce apoptosis in these cells.

Together these data demonstrate that there are significant differences in the survival characteristics of monocytes and Mø. Monocytes undergo significant constitutive apoptosis, which can be increased by serum depletion and by loss of adherence, whereas Mø, which have a low level of constitutive apoptosis, are not influenced by these factors. Given the

complexity of human serum, the use of serum free media and of blocking antibodies could help determine the relative contribution of various factors to monocyte and Mø survival, as would a measure of the relative levels of synthesis and secretion of these growth factors by both monocytes and Mø. Supernatant transfer experiments could be performed between Mø and monocyte cultures to indicate whether important levels of survival factors were being secreted by Mø. Of the range of cytokines investigated, none increased monocyte apoptosis and only TNF-α promoted monocyte and murine bone marrow derived Mø survival; none had any influence on human Mø longevity. TNF-α in combination with cycloheximide exerted the opposite effect, significantly augmenting apoptosis. Monocyte survival characteristics were more in line with those of U937 cells and murine bone marrow derived Mø whilst alveolar Mø and monocyte derived Mø were similar in their resistance to apoptosis and prolonged survival. One possible interpretation of these data is that susceptibility to apoptosis is related to changes in the cell cycle controls in monocyte and Mø populations, associated with differentiation, a suggestion that is explored further in Chapter 6.

Chapter 5

CHANGES IN CYTOKINE RECEPTOR EXPRESSION ASSOCIATED WITH MACROPHAGE MATURATION AND APOPTOSIS.

5. 1 INTRODUCTION.

The Mø is capable of both secreting and responding to a wide range of cytokines (Gordon, 1992 and 1995). The pattern of secretion changes according to cell factors such as priming and environmental factors such as the presence of inflammation. Likewise the pattern of Mø responses to cytokines alters depending upon a number of factors including the activation state, and the degree of differentiation (Gordon, 1995). Several mechanisms may alter the way Mø respond to cytokines, including the expression of receptors for specific cytokines, the functional capacity of these receptors and the intracellular environment which can affect signal transduction (Miyajima, 1992a). Cytokine receptor expression is known to be dynamic, changing with temperature, activation state and maturity; furthermore receptor shedding is now recognized to be a common event (Mullberg, 1995). In the light of the differences in survival characteristics of monocytes and Mø, I sought to investigate the expression of cytokine receptors during the process of differentiation from monocyte into Mø and with the onset of apoptosis itself. Patterns of altered cytokine receptor expression may be restricted to specific cytokine receptor families. Moreover, although considerable progress has been made in the elucidation of the molecules involved in Mø recognition of apoptotic cells, the nature of the changes on the apoptotic cell surface which signal phagocytic responses have not been clearly determined. Investigation of the surface changes associated with apoptosis in a number of different cell types, including monocytes, Mø, monocyte-like cell lines and PMN may thus be particularly revealing.

5. 2 RESULTS.

5. 2. 1 Cytokine Receptor Expression on Human Monocytes, Macrophages and U937 Cells and the Changes Accompanying Apoptosis.

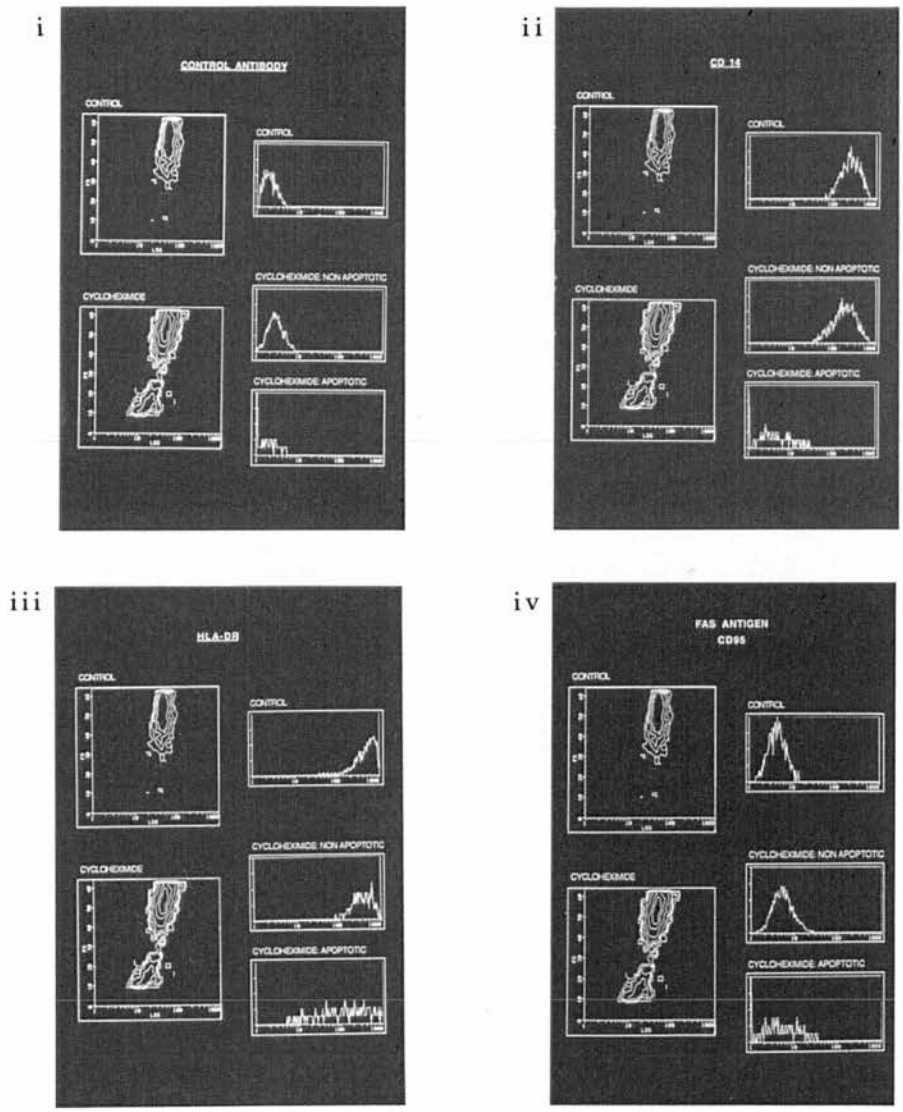
As a participant in the cytokine receptor section of the Fifth International Workshop and Conference on White Cell Differentiation Antigens I received a panel of 72 mAb of known and unknown specificity (Kikutani, 1995). Of these 72 mAb, 3 were withdrawn (C008, C035 and C036) and 5 were received too late to be included (C073, C074, C075, C076 and C079). Thus, I present the data on the remaining 64 antibodies which were tested by flow cytometry on human peripheral blood monocytes, monocyte derived Mø, U937 cells and human PMN. For all these cell types, cytokine receptor expression on both non-apoptotic and apoptotic cells was determined, apoptosis being induced by three different treatments, cycloheximide for Mø, serum withdrawal for U937 cells and aging in culture for PMN.

Fifty one of the mAb provided were raised against either recombinant molecules or transfectants expressing specific receptors, hence their specificity's were known, the remaining 13 were of unknown specificity. At the conference results from all the participating groups were collated and on that basis new CD numbers (and in cases of inconclusive evidence, provisional CDw numbers) were allocated to specific cytokine receptors. Fifty three of the cytokine receptor panel mAb were clustered (i.e. assigned CD numbers), including all the 51 mAb of known specificity and 2 with previously unknown specificity, 11 mAb remained unclustered (Kikutani, 1995). Panel mAb were identified by the letter C and a three digit number, the mAb tested are listed in Appendix 2

showing their cytokine panel code and the CD (or CDw) number they were ascribed at the conference.

To determine human monocyte and Mø cytokine receptor expression using this panel of mAb I isolated cells as described in section 2. 3. 1. A proportion of monocytes from each donor was used to determine cytokine receptor expression and the remainder were matured into Mø. Mø apoptosis was induced by treatment with 50 μM cycloheximide for 14 hours. Apoptotic Mø (10 - 40% of the population) could be distinguished from non-apoptotic cycloheximide treated Mø by flow (section 3. 2. 8). By gating on these two sub-populations, the expression of cytokine receptors and the changes induced by apoptosis could be determined using indirect immunofluorescence. Comparison of cytokine receptor expression on control Mø (cultured for 14 hours in identical conditions but without the addition of cycloheximide) with the non-apoptotic Mø sub-population exposed to cycloheximide allowed the effect of cycloheximide alone to be determined. Thus, the relative effects of protein synthesis inhibition and apoptosis on human Mø cytokine receptor expression could be distinguished (**Figure 5. 1**). Furthermore, comparison with monocytes allowed changes in cytokine receptor expression occurring during *in vitro* differentiation into Mø to be assessed. Apoptosis was induced in the myelomonocytic U937 cell line by serum withdrawal and again flow cytometry was used to discriminate between the large non-apoptotic and small apoptotic cells (section 4. 2. 1 and **Figure 4. 1**) and their individual cytokine receptor profiles could be determined. A summary of these results is provided in **Table 5. 1**. **Appendix 2** shows the results of this study in full, providing the mean fluorescence for each mAb tested within each CD group relative to the isotype.

Figure 5. 1 Determining Changes in Surface Receptor Expression of Macrophages with Apoptosis using Flow Cytometry.



Legend for Figure 5. 1 Binding of of **i)** Isotype control mAb, **ii)** anti-CD14 mAb, **iii)** anti-HLA-DR mAb and **iv)** anti-Fas (CD95) mAb to - control Mø and - cycloheximide treated Mø. Cyloheximide treatment induced apoptosis in a proportion of cells as seen by the forward and side scatter flow profiles. By specifically gating on non-apoptotic cycloheximide treated Mø and apoptotic Mø and comparing this with receptor expression on control Mø, the effect of apoptosis and protein synthesis inhibition on receptor expression on Mø could be demonstrated.

Table 5. 1

Summary of Cytokine Receptor Expression on Monocytes, Changes with Differentiation and Apoptosis; Comparison with U937 Cells.

Cytokine	Mono	Macrophages			U937	
Receptor		Control	CHX	Apop	non-Apop	Apop
GM-CSFR	+	++	++	+	+	±
SCFR (c-*kit*)	-	+	ND	ND	+	±
IFNγR	+	+	+	+	+	+
TNFR p55	-	+	±	-	+	±
TNFR p75	+	++	±	-	+	-
IL-1R type 1	-	+	ND	ND	++	+
IL-1R type 2	+	++	++	±	+	±
IL-2Rα	-	+	+	+	+	+
IL-2Rβ	-	±	±	-	+	-
IL-4R	-	+	+	-	-	-
IL-6R	±	±	±	-	+	-
gp130	-	-	ND	ND	±	±
Fas Antigen	+	+	+	+	+	+
CDw 84	+	++	+	-	±	±

Legend for Table 5. 1

A summary of the cytokine receptor expression of monocytes, Mø, U937 cells and changes induced by apoptosis:

- = No expression.

± = Weak expression.

\+ = Significant expression.

++ = Significant increase from already positive value.

ND = Test not done. (IL-7 or IL-8 receptors could not be detected).

5. 2. 2 Changes in Cytokine Receptor Expression on Human Polymorphonuclear Leukocytes Accompanying Apoptosis.

The cytokine receptor profile of human PMN was examined and changes with apoptosis determined (see section 2. 7. 2. 3a) to see if the pattern of changes related with that of monocytic cells. This was important as PMN underwent apoptosis constitutively whilst Mø and U937 cells required active intervention to induce apoptosis. Twenty four of the cytokine receptor panel Ab were tested on aged PMN but only 6 cytokine receptor groups were positive; IL-1R types 1 and 2 (CD 121a and 121b), GM-CSFR (CDw 116), TNFR p55 and p75 (CD120a and 120b) and the Fas antigen (CD95). Two groups, IL-6R (CD126) and IL-8R (CDw 128) had one positive and one negative mAb and the IL-4R was weakly positive. Of the mAb with positive binding only those against CD120b showed significant decline on apoptotic PMN although there was a slight decline in CD120a expression. The results are presented in detail in **Appendix 3**, with an adjacent summary reviewing the changes in receptor expression on PMN, Mø and U937 cells as they undergo apoptosis.

5. 2. 3 Anti-Fas Antibody Does Not Induce Macrophage Apoptosis.

Fas, a member of the TNFR superfamily, has been shown to transduce an apoptotic signal. Antibodies against Fas induce apoptosis in lymphocytes (Itoh, 1991; Oehm, 1992). I tested the anti-Fas murine mAb C071 on human Mø to determine whether apoptosis would be induced. As a control three other murine mAb, not known to induce apoptosis were used, an IgM anti-CD25 isotype control and two IgG mAb, an anti-CDw 84 mAb which binds strongly and anti-IL-6R mAb which binds weakly to Mø. None of the mAb tested had any effect on human Mø apoptosis, results are presented in **Table 5. 4**.

Table 5. 4

Anti-Fas Antibody does Not Induce Macrophage Apoptosis.

Treatment	% Apoptosis		p
	Non-Adherent	Adherent	value
Control	7.3 ± 1.6	2.0 ± 0.7	-
Anti-Fas	4.9 ± 2.1	2.9 ± 0.3	n/s
Anti-CDw 84	4.6 ± 1.9	1.3 ± 0.8	n/s
Anti-IL-6R	6.2 ± 0.9	1.1 ± 0.9	n/s
Anti-CD25	3.1 ± 2.0	2.8 ± 0.4	n/s
50 mM CHX	16.8 ± 1.5	5.5 ± 0.8	$p<0.01$

Legend for Table 5. 4

Human monocyte derived Mø (0.5×10^6/ml) cultured in 10% autologous serum were incubated with medium (Control) or with 0.5 and 50 μg/ml anti-Fas monoclonal antibody (Anti-Fas) or similar concentrations of anti-IL6R (Anti-IL-6R), anti CDw 84 (Anti CDw 84) or an isotype control anti-CD25 monoclonal antibody MEM 141 (Anti-CD25). As a positive control for apoptosis, Mø were incubated in the presence of 50 μM cycloheximide. All cells were incubated in flexiwell 96 well trays in standard conditions for 14 hours, duplicate wells were prepared for each treatment. Non-adherent cells were washed off and adherent cells lifted off by 5 minutes treatment with 5 mM ice cold EDTA. Cell recoveries were all within 10% of starting numbers, non-adherent cells were <5% total for all treatments except

CHX where 9.5 ± 0.9% of cells were non-adherent. Apoptosis was assessed by flow cytometry and confirmed by acridine orange fluorescent microscopy. Results were the same for 5 μg/ml and 50 μg/ml anti-Fas and are presented for 50 μg/ml only. Results are expressed as mean ± SE, (n = 3). The p value is shown for the comparison between non-adherent Control and the non-adherent cells from the other treatments but the same results pertain for adherent cells.

5. 3 DISCUSSION.

Using the panel of monoclonal antibodies against a range of cytokine receptors provided by the Cytokine Receptor Section of the 5th International Workshop on Leukocyte Differentiation Antigens, a comprehensive picture was obtained of the changing pattern of expression of cytokine receptors accompanying human Mø maturation and apoptosis (Bellingan, 1993 and 1995). Freshly isolated human monocytes were shown by flow cytometry to express GM-CSFR (CDw 116), IFNγR (CDw 119), TNFR75 (CD120b), IL-1R type 2 (CDw 121b) and IL-6R (CD126) as well as Fas antigen (CD95) and CDw 84. They did not express the following receptors at detectable levels by conventional flow cytometry: SCFR (CD117), TNFR55 (CD120a), IL-1R type 1 (CDw 121a), IL-2R α or β chains (CD25 and CD122), IL-4R (CDw 124), IL-7R (CDw 127), IL-8R (CDw 128) or gp130 (CDw 130). During *in vitro* maturation Mø gain expression of SCFR, TNFR55, IL-1R type 1, IL-2R α and β chains and IL-4R and show increased expression of GM-CSFR, TNFR75 and IL-1R type 2. Of the range of receptors examined, only IL-7R, IL-8R and gp 130 cannot be detected by flow cytometry on monocytes and Mø, implying that expression is less than one thousand molecules per cell (Zola, 1990 and

1995). Expression of IFN-γR, IL-6R and the Fas antigen remain unchanged. Apoptosis, induced by cycloheximide, resulted in significant loss of expression of a number of receptors namely: GM-CSFR, TNFR55 and TNFR75, IL-1R type 2, IL-2R (β chain only), IL-4R, IL-6R, and CDw 84. Importantly apoptosis does not induce loss of all receptors, with unchanged expression of the Fas antigen, the α chain of the IL-2R and the IFN-γR. The observed specific loss of receptors associated with apoptosis suggests that this may contribute to functional isolation of apoptotic cells which may be important in limiting inflammation. In keeping with this suggestion none of the known cytokine receptors tested were up-regulated with Mø apoptosis. Protein synthesis inhibition alone results in significant loss of expression of both types of TNFR, IL-4R and CDw 84 but does not have a global effect, with GM-CSFR, IFNγR, IL-1R type 2, IL-2R α and β chains, IL-6R and Fas antigen all remaining unaffected. This observation may relate to the levels of receptor turnover in the cell, with rapidly recycled receptors being lost following cycloheximide treatment. In relation to specific cytokine receptor families, no pattern of changes emerged.

U937 cells constitutively expressed all the receptors tested except IL-4R, IL-7R and IL-8R, they had very low expression of gp 130 and CDw 84. Apoptosis was induced in these cells by serum withdrawal, this resulted in the reduction in expression of GM-CSFR, SCFR, TNFR55, IL-1R types 1 and 2 and the complete loss of TNFR75, IL-2R β chain and IL-6R. Again apoptotic U937 cells retain expression of the Fas antigen, IFNγR and IL-2R α chain. These changes with apoptosis are exactly the same as those undergone by apoptotic Mø, despite the different system of induction.

Although other groups, using exactly the same mAb, determined cytokine receptor expression on monocytes and U937 cells, none followed monocytes during maturation to Mø and Mø death (Agis, 1995; Ross, 1995). At the workshop 465 antibodies, including a representative monoclonal antibody from all the cytokine receptor CD classifications, were analyzed as part of a Blind Panel (Shaw, 1995). This Blind Panel analysis was performed by flow cytometry in 28 laboratories who determined expression of the relevant antigens on over 80 different cell types. In the section below I compare my determination of monocyte and U937 cytokine receptor expression with those obtained by the Blind Panel and other groups, showing close agreement particularly for the monocyte. It is notable that the level of expression of most cytokine receptors are very low when compared with other Mø surface molecules such as CD14 or HLA-DR, as shown by the controls.

There are three IL-2R isoforms with high, intermediate and low binding affinities for IL-2 (Smith, 1988). The high affinity IL-2R consists of an $\alpha\beta\gamma$ trimolecular complex, the intermediate affinity form is a $\beta\gamma$ heterodimer complex and the low affinity form consists of the α chain alone (Ishii, 1995). Three different epitopes to CD25 (IL-2Rα) have been recognized (Sun, 1995). CD25 is expressed mainly on lymphoid cells, in particular on certain T cells and on NK cells. I did not detect significant binding of any of the 5 monoclonal antibodies against IL-2R α chain on monocytes but U937 cells did express the IL-2R α chain which fell significantly with apoptosis. Likewise none of the Blind Panel detected IL-2Rα expression on monocytes although Ross *et al.* and Agis *et al.* found activated monocytes to be very weakly positive (Agis, 1995; Ross, 1995). The Blind Panel did not detect IL-2R α chain on U937 cells.

The IL-2R β chain (CD122) is strongly expressed on NK cells but only weakly on other lymphocytes (Autschbach, 1995). I did not detect any expression of the IL-2R β chain on monocytes with any of the 7 monoclonal antibodies used, nor did the Blind Panel or Ross *et al.* , however 2 groups did find monocytes to be very weakly positive (Agis, 1995; Ishii, 1995). Although I did not detect any expression on monocytes, these molecules were expressed on Mø and U937 cells, although both at very low levels. Expression of the β chain fell with Mø and U937 cell apoptosis. The Blind Panel did not detect binding on U937 cells, possibly reflecting minor variations in cell phenotype between laboratories.

There are 2 classes of GM-CSFR, a low affinity receptor consisting of the α subunit alone and a high affinity receptor, a heterodimer of both α and β subunits (reviewed in Miyajima, 1992a). The β subunit, which is common to the IL-3 and IL-5 receptors (Kitamura, 1991 and 1995), enhances binding of the α subunit to the cytokine and facilitates signal transduction. Early haematopoietic stem cells express GM-CSFR, this is increased in those cells committed to the myeloid lineage and decreased in committed lymphoid cells. (Olweus, 1995). All groups at the workshop found monocytes and U937 cells to express GM-CSFR. These data show that with maturation into Mø the expression of GM-CSFR is significantly increased. Inhibition of protein synthesis does not alter GM-CSFR expression but apoptotic Mø and U937 cells have nearly a 4 fold reduction in GM-CSFR expression.

The proto-oncogene *c-kit* encodes the tyrosine kinase transmembrane SCFR, other members of this family of receptors include CSF-1 and PDGF

receptors types A and B. Only a minority of bone marrow cells express SCFR (1-4%), most of these co-express CD34 and represent the progenitor cells for all haemopoietic lineages (Buhring, 1995). Neither I nor any of the other groups detected any SCFR expression on monocytes, U937 cells did express SCFR and apoptosis led to a small but significant fall in expression.

IFNγ is known to be a differentiation factor for Mø and all groups confirmed monocytes to be 100% positive for IFNγR, although with less than 10, 000 receptors per cell. Expression of the IFN-γR was not significantly altered during monocyte to Mø differentiation, protein synthesis inhibition or with apoptosis. In line with the Blind Panel I found that U937 cells also expressed the IFNγ receptor, with no significant change in expression during apoptosis.

Although the TNF55R is believed to be the main receptor mediating TNF-α induced cytotoxicity, both receptors are required for superoxide production and recent reports demonstrate that both TNF receptors are important for the induction of apoptosis (Grell, 1994; Richter, 1995; Vandenabeele, 1995). Mice deficient in the TNF55R are resistant to endotoxic shock but susceptible to intracellular pathogens, deletion of the TNF75R has less obvious effects (Beutler, 1994; Pfeffer, 1993). These receptors show significant homology in their extracellular domains but their cytoplasmic regions are different and they utilize distinct signal transduction pathways (Grell, 1994). Using Ficoll-prepared mononuclear cells none of the Blind Panel showed any positive TNF55R expression, although with whole blood preparations some laboratories showed weak TNF55R antibody binding by monocytes. This may represent loss of

receptor expression during cell isolation or a sensitivity problem with many cytokine receptors expressed at very low levels on quiescent cells, requiring high sensitivity flow cytometry to demonstrate expression (Zola, 1990 and 1995). In contrast both this study and Agis *et al.* and Ross *et al.* demonstrate that the TNFR75 is expressed by monocytes (Agis, 1995; Ross, 1995). Expression of both receptors increased with maturation into Mø whilst protein synthesis inhibition and apoptosis both independently caused a significant loss of expression of both types of receptor. All groups found U937 cells expressed both the TNF55R and the TNF75R and I showed that, as with Mø, expression of both fell significantly during U937 cell apoptosis. TNFR are also known to be shed rapidly from PMN (Ding, 1992) and monocytes (Leeuwenberg, 1995) when activated, being proteolytically cleaved and forming soluble proteins that retain TNF binding capacity (Engelmann, 1989). Whether this generation of soluble TNFR represents a protective response is as yet unknown.

There are two types of IL-1R, type 1 which transduces signals and type 2 with a short cytoplasmic region that does not, both have three immunoglobulin domains. The type 1 receptor is known to be weakly expressed on Mø, certain lymphocytes and endothelial cells (Kikutani, 1995) whilst the type 2 receptor is expressed on a subset of B cells, and on monocytes (Zola, 1995; Agis, 1995). I demonstrated expression of IL-1R type 2 but not type 1 on monocytes in accordance with all other investigators at the workshop, with both types of IL-1R increased during maturation into Mø, especially the type 2 receptor. Expression of IL-1R by Mø was unchanged by protein synthesis inhibition but fell markedly with apoptosis, which together with data for loss of these receptors with

apoptosis on U937 cells suggests specific changes associated with cell death.

In agreement with Agis *et al.* and the Blind Panel, I did not detect IL-4R expression on monocytes (Agis, 1995) or on U937 cells. Mø expressed IL-4R and lost this expression with apoptosis but not with protein synthesis inhibition.

Competitive binding studies using purified IL-6 receptor protein suggests at least 5 epitopes on the IL-6R as shown in **Table 5. 5** (Gaillard, 1995).

Table 5. 5

Epitope Mapping of the IL-6R by Cytokine Receptor Panel Antibodies.

Epitopes				
1	2	3	4	5
C014	C019	C015	C020	C059
C066		C016	C040	C061
		C017	C060	C064
		C018	C067	C065
		C058		
		C062		

Monoclonal C063 was not classified.

Binding of all the anti-IL6 receptor mAb to monocytes was weak (except C060 - see later) and showed no significant increase on Mø, with no apparent differences between epitope groupings, a finding confirmed by Ross *et al.* and the Blind Panel (Ross, 1995). Unexpectedly, C060 showed very positive binding to monocytes and Mø. This antibody falls within

epitope group 4, the mAb in this group showing weak binding, as found for other anti-IL-6R mAb. Agis *et al.* also reported C060 to be the only anti-IL-6R monoclonal antibody with which they detected binding to elutriated monocytes (Agis, 1995). In agreement with the blind panel I found U937 cells expressed significant levels of IL-6R, which fell significantly with apoptosis. Expression of C060 was not high on U937 cells. IL-6 signal is transduced by gp130 which is also associated with other receptors including IL-11 and thus has a broader tissue distribution than IL-6R alone. Interestingly, despite demonstrating IL-6R expression on monocytes I did not detect expression of gp130, likewise, the Blind Panel and Agis *et al.* found monocytes to be negative for gp130 (Agis, 1995), all groups found U937 cells expressed low levels of gp130.

I did not detect IL-7R or IL-8R expression on monocytes or U937 cells, nor did Agis *et al.* , Morohashi *et al.* or the Blind Panel (Agis, 1995; Morohashi, 1995). Ross *et al.* showed 9% of monocytes bound anti-IL-7R antibody and this increased to 36% with LPS activation, they did not detect any IL-8R expression on monocytes (Ross, 1995).

The antigens recognized by anti-APO-1 and anti-Fas are the same (Oehm, 1992; Iwai, 1994). This glycoprotein molecule has three cysteine rich extracellular domains and is a member of the TNFR superfamily (Eischen, 1994; Mallett, 1991). The Fas antigen is widely expressed on cells, including T and B lymphocytes, some NK cells, PMN, monocytes and endothelial cells; platelets do not express it (Iwai, 1994; Richardson, 1994; Robertson, 1995). Three monoclonals against Fas antigen were tested by the Blind Panel, all were weakly positive on monocytes and U937 cells, consistent with my findings. Expression of CD95 remained

unchanged on Mø and apoptotic Mø and likewise expression did not fall significantly on apoptotic U937 cells. In keeping with Robertson *et al.* and Iwai *et al.* (Iwai, 1994; Robertson, 1995), I did not demonstrate any increase in Mø apoptosis when incubated with the anti-Fas monoclonal antibody. Fas has an important role in T cell selection, defects resulting in loss of Fas expression are seen in mice homozygous for *lpr* (lymphoproliferation) and cause a striking degree of lymphadenopathy (Watanabe-Fukunaga, 1992). Antibodies against the Fas antigen are known to induce apoptosis (Itoh, 1991; Oehm, 1992), including in activated lymphocytes, suggesting a role for Fas antigen in clonal deletion of autoreactive T cells (Lenardo, 1991; Nishimura, 1995). Fas interaction with its natural ligand, Fas ligand, also results in apoptosis; Fas ligand is expressed on activated T cells and the interaction of Fas with this ligand results in apoptotic death of these activated T cells (Liles, 1996). Cytotoxic T cells express Fas ligand and can kill target cells through Fas ligand/Fas interactions (Lowin, 1994). There is greater controversy about the role of Fas on other cell types and whether cell activation is required for the induction of apoptosis. Richardson *et al.* reported that 3 μg/ml anti-Fas mAb triggered apoptosis in up to 30% of Mø but not in endothelial cells (Richardson, 1994). However both Robertson *et al.* and Liles *et al.* demonstrated that monocyte viability was not affected by anti-Fas antibodies (Robertson, 1995; Liles, 1996). Iwai *et al.* using 0.03 to 3 μg/ml anti-Fas mAb could not detect anti-Fas mediated apoptosis on unactivated lymphocytes and only detected very low levels of DNA fragmentation in monocytes. In contrast, anti-Fas antibodies caused a significant increase in PMN apoptosis (Iwai, 1994). Recently, ligation of Fas has been shown to induce ROI formation, suggesting that cells with increased anti-oxidant protective mechanisms may be resistant to Fas

mediated apoptosis (Kohno, 1996). This observation raises the possibility that the requirement for prior activation for Fas mediated cell death may be a ROI mediated phenomenon and that the balance of oxidants-anti-oxidants can be tipped towards ROI excess by ligation of the Fas ligand. Whether the observed lack of effect of Fas upon Mø apoptosis reflects lack of cellular activation or is related to the capacity of the Mø anti-oxidant mechanisms is unclear at present.

Of the antibodies against unknown antigens submitted to the Cytokine Receptor section of the Workshop, two (C056 and C057) were clustered along with one other antibody submitted to the B-cell section, into a provisional CD classification CDw 84 recognizing a molecule of unknown function. These two antibodies demonstrated a unique pattern of expression with low level of binding to B and T-cells whilst monocytes Mø and platelets were all strikingly positive. I observed a very marked increase in expression during Mø maturation, with marked reductions following apoptosis.

None of the other antibodies against unknown antigens were clustered and apart from C078, these unclustered antibodies did not show significant reactivity with monocytes or Mø; C078 binding fell with Mø maturation.

Thus the cytokine receptor expression on monocytes, determined in this chapter, is closely in accord with that found by other workers using the same mAb. U937 cells expressed a very wide range of cytokine receptors including GM-CSFR, IFNγR, TNFR55 and 75, IL-1R type 2, IL-6R, gp 130, Fas antigen, and CDw 84 as well as lacking expression of IL-4R, IL-7R and

IL-8R. There were certain differences between my data and results reported at the Boston workshop, with my data showing U937 cell expression of IL-2R α and β chains, SCFR and IL-1R type 1. Whether this indicates an outgrowth of a slightly different strain of this cell line is not clear. Certainly these cells, with typical monocytic cell morphology, were shown to express CD64 (Fc RI), HLA-DR and CD14 and to be negative for CD3 and CD16, all consistent with U937 lineage. Changes in cytokine receptor expression induced on U937 cells with apoptosis were found to be the same as that for Mø, despite the different method of inducing apoptosis. Importantly this observation extends also to those receptors that are retained (Fas antigen, IFNγR and IL-2R α chain). In addition both Mø and U937 cells are seen to lose CD14 and HLA-DR with apoptosis.

Of the antibodies against unknown antigens, several bound to U937 cells but not to Mø, including C023, C024, C030, C031, C034, C047 and C078. There was a significant increase in the binding of C032, C033 and C077 on apoptotic U937 cells, although it is unlikely that this represents new expression of apoptosis-related proteins. Since apoptotic monocytes and PMN did not show increased binding of C032, C033 and C077, expression does not appear to be a general feature of apoptotic cell death. Secondly, apoptotic U937 cells were trypan blue positive and bind the mAb BOB78, an antibody which recognizes an intracellular antigen (Dr. I. Dransfield, personal communication). This suggests that the increased binding of these mAb may reflect an early change in the membrane permeability characteristics of apoptotic U937 cells which exposes cytoplasmic antigens.

Human PMN were shown to express GM-CSFR, TNF55R and TNF75R, IL-1R type 1 and 2, IL-6R, IL-8R and Fas antigen. The Blind Panel results

were very similar although they did not demonstrate any binding to TNF55R or IL-1R type 1. As TNF-α promotes PMN apoptosis and recent work suggests that both receptors are required for the apoptotic signal to be effected, it is likely that PMN do express the TNF55R even if only at very low levels. Differences in cell isolation methods may account for differences between my results and those of the Blind Panel. PMN, like Mø and U937 cells lose expression of both TNFR types with apoptosis but retain expression of the Fas Antigen. Unlike apoptotic Mø and U937 cells, apoptotic PMN also retain expression of GM-CSFR and both types of IL-1R. I demonstrated that only one of the IL-8R antibodies (C070) bound PMN, this was confirmed by Morohashi *et al.* who showed that there was no positive immunofluorescence on any peripheral blood cells for C069 but that C070 strongly stained PMN and weakly stained lymphocytes but did not stain monocytes (Morohashi, 1995).

This work emphasizes that similar changes in surface molecule expression are observed for apoptotic cell types irrespective of the method of induction of apoptosis. Notably the two types of TNFR are rapidly lost from the surface of the apoptotic cells, whilst others, particularly the Fas antigen are retained. Whether there is any functional significance associated with maintained expression of these receptors is as yet unknown.

The mechanism of receptor loss with apoptosis is unknown; they may be shed, suggesting activation of a proteolytic mechanism, or they could be internalized. In the case of the Mø, protein synthesis inhibition alone cannot explain the observed changes as non-apoptotic Mø exposed to cycloheximide still retained significant cytokine receptor expression.

Receptor internalization is unlikely to account for altered receptor expression, indeed for apoptotic U937 cells receptor expression fell with apoptosis despite cell permeabilization. Receptor shedding is perhaps the most likely explanation of receptor loss, suggesting activation of a proteolytic mechanism. Proteolytic cleavage of TNF and IL-6 receptors with activation has been shown in other cells (Mullberg, 1995; Williams, 1996) and CD14 (Bellingan, 1995) and CD16 (Dransfield, 1994) loss with apoptosis clearly demonstrated. Future analysis of soluble receptor levels in cell supernatants, to determine the degree to which shedding is involved, would be of interest.

In conclusion, this work demonstrates an increased cytokine receptor repertoire accompanying monocyte to Mø differentiation consistent with their increased functional capacity. With the induction of apoptosis many, but not all, of these receptors are lost, possibly by receptor shedding. Apoptosis associated changes were found to be common to other cell types (U937 and PMN) and independent of the method of induction of apoptosis. Certain receptors are retained late into the apoptotic process including the Fas antigen demonstrating that loss of surface molecule expression is not a universal event.

5. 4 Appendix 2

Cytokine Receptor Expression of Human Monocytes and the Changes with Differentiation into Macrophages and Apoptosis; Comparison with U937 Cells and their Changes with Apoptosis.

CD25 (IL-2 receptor α)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	2	6	3	3	3	3
C003	1.01	1.40	1.37	1.15	1.70	1.37
C010	1.12	1.64	-	-	8.93	2.50¶
C012	1.05	1.16	-	-	1.57	1.07
C052	1.08	2.50	1.0	1.27	7.10	1.70
C068	1.02	1.16	-	-	1.0	1.00
Mean	1.05	1.57§	1.19	1.21	4.06	1.53¶

CDw 116 (GM-CSF receptor)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	3	8	6	6	3	3
C007	2.84	5.79§	3.39	1.49†*	3.23	1.27¶

CD117 (SCF receptor or *c-kit*)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	2	4	-	-	3	3
C009	1.06	1.11	-	-	2.03	1.40¶
C021	1.02	1.18	-	-	2.07	1.30¶
C055	1.01	1.10	-	-	2.57	1.50¶
C022	1.21	1.31	-	-	2.83	3.40
C054	1.06	1.16	-	-	2.93	4.30¶
C072	1.00	1.41	-	-	3.60	1.40
Mean	1.06	1.21§	-	-	2.67	2.20¶

CDw 119 (IFNγR)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	2	4	3	3	3	3
C049	1.25	2.75	1.50	1.33	2.70	1.60

CD120a (TNFR type 1 or p55)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	3	6	5	5	3	3
C025	1.16	2.55§	1.77	1.33†*	1.80	1.10
C026	1.03	2.17§	1.40#	1.13†*	1.80	1.20
C027	1.03	1.88§	1.22#	1.11*	1.80	1.30
C028	1.04	2.24	1.42#	1.13*	2.10	1.70
C042	1.02	1.64§	1.02#	1.06*	1.70	1.30
Mean	1.06	2.10§	1.37#	1.15†*	1.84	1.32¶

CD120b (TNFR type 2 or p75)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	3	9	9	9	3	3
C006	1.93	3.75§	3.01	1.23†*	3.83	1.43
C029	1.77	4.87§	1.37#	1.01*	4.20	1.30¶
C043	1.41	3.28	1.08#	1.06*	2.50	1.20¶
Mean	1.70	3.97§	1.82#	1.10*	3.51	1.31¶

CDw 121a (IL-1R type 1)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	3	4	-	-	3	3
C001	1.06	1.40§	-	-	4.20	2.40¶

CDw 121b (IL-1R type 2)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	3	8	7	7	3	3
C002	1.90	4.45§	4.39	1.67†*	4.30	1.50¶

CD122 (IL-2 receptor β)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	3	6	4	4	3	3
C044	1.00	1.07	-	-	4.10	1.50¶
C045	1.11	1.65	-	-	5.40	1.35
C046	1.16	1.18	1.17	1.20	2.80	1.25¶
C048	1.05	1.52§	1.50	1.15†	4.50	1.45¶
C051	1.01	1.20	-	-	1.00	1.15
C050	1.01	1.14	-	-	2.80	1.15
C053	1.01	1.15	1.00	1.09	1.30	1.05
Mean	1.05	1.27§	1.22	1.15	3.10	1.27¶

CDw 124 (IL-4R)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	3	8	6	6	3	3
C004	1.09	1.77	1.46	1.13*	1.03	1.70

CD126 (IL-6R)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	3	7	6	6	3	3
C014	1.22	1.56	-	-	3.63	1.30
C015	1.41	1.60	1.28	1.43	7.57	1.80¶
C016	1.20	1.57	-	-	5.43	1.50¶
C017	1.18	1.39	-	-	6.17	1.50¶
C018	1.19	1.71	1.45	1.43	8.60	2.20¶
C019	1.52	1.91	1.34	1.21	9.90	1.90¶
C020	1.38	1.84	1.50	1.32	9.20	2.00¶
C040	1.35	1.36	1.07	1.04	3.27	1.40¶
C058	1.37	1.46	1.46	1.09	3.80	1.20
C059	1.45	1.45	1.23	1.08†*	3.20	1.20
C060	8.38	21.6§	16.4	5.92†*	4.40	1.40¶
C061	1.48	1.32	1.11	1.14	3.97	1.30¶
C062	1.44	1.48	1.01#	1.05*	3.80	1.30
C063	1.10	1.27	-	-	4.10	1.40¶
C064	1.34	1.31	1.17	1.09	3.50	1.10
C065	1.39	1.34	1.15	1.06	3.30	1.10
C066	1.12	1.47	1.00	1.00	3.60	1.20
C067	1.04	1.09	-	-	1.80	1.00
Mean -C060	1.30	1.48	1.23	1.16*	4.96	1.43¶
Mean +C060	1.69	2.59	1.36	1.53*	4.91	1.32¶

CDw 127 (IL-7R)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	2	4	2	2	3	3
C005	1.08	1.58	1.34	1.12	1.07	1.20

CDw 128 (IL-8R)

Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	2	3	-	-	3	3
C069	1.00	1.05	-	-	1.10	1.70
C070	1.04	1.24	-	-	1.00	1.00

CDw 130 (gp130)						
Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	2	3	-	-	3	3
C041	1.13	1.16	-	-	1.27	1.23

CD95 (Anti-Fas)						
Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	3	9	7	7	3	3
C071	1.96	2.92	2.69	2.53	3.80	2.20

CDw 84 (unknown function)						
Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	2	8	7	7	3	3
C056	4.63	31.1§	23.0#	3.86†*	1.30	1.10
C057	4.87	34.2§	24.2#	4.40†*	1.40	1.20

Unclustered at the workshop						
Panel Code	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	2	3	3	3	3	3
C011	1.01	1.06	-	-	1.07	1.40
C023	1.47	2.00	1.19	1.97	2.93	1.33¶
C024	1.19	1.21	-	-	5.57	2.87¶
C030	1.06	1.34§	1.30	1.19	3.60	2.47
C031	1.47	1.71	-	-	9.10	2.20¶
C032	1.01	1.10	-	-	1.00	1.53¶
C033	1.03	1.21	-	-	1.00	45.3¶
C034	1.17	1.99	-	-	8.53	1.80¶
C047	1.01	1.22	-	-	2.53	1.90
C077	1.00	1.51	2.09	1.24	1.00	2.30¶
C078	4.69	1.87	1.33	1.52	4.50	1.45¶

Controls						
Antigen	Mono	MØ	CHX MØ	Apoptotic MØ	U937	Apop U937
No.	3	7	7	7	3	3
CD14	104	71§	44#	7.8*†	6.8	1.7¶
HLA-DR	32	151§	135	51*†	5.9	1.7¶
BOB 78	-	-	-	-	1.45	7.2¶

Legend for Table 5. 2
Results of binding of panel of cytokine receptor mAb, listed by the cytokine receptor panel code (Panel Code) to human peripheral blood

monocytes (Mono), macrophages (MØ), cycloheximide treated non-apoptotic macrophages (CHX MØ), cycloheximide treated apoptotic macrophages (Apoptotic MØ), U937 cells (U937) and apoptotic U937 cells (Apop U937). Apoptosis was induced by cycloheximide (50 μM) in Mø and by serum withdrawal in U937 cells. mAb binding was determined by indirect immunofluorescence, gating on the non-apoptotic and apoptotic populations which were distinguished by forward and side scatter flow cytometry parameters. The number of donors tested for each cytokine receptor and for each cell type are shown (No.). If a test has not been performed this is indicated by a (-). Results are expressed as relative mean fluorescence, determining the logarithmic fluorescence value for each antibody and dividing this by that of a non-binding control MOPC-21C. A value of 1.00 indicates no difference between non-binding control and test mAb. Positive controls were provided by analyzing binding of mAb against the known Mø associated antigens - CD14 and HLA-DR (see also section 3. 2. 9). The mAb BOB 78 binds to an intracellular antigen. For each mAb, changes in binding between the following were determined:
1) monocytes and control untreated Mø,
2) cycloheximide treated non-apoptotic and apoptotic Mø,
3) control Mø and apoptotic Mø,
4) control Mø and cycloheximide treated non-apoptotic Mø,
5) Non apoptotic and apoptotic U937 cells.

Significance of $p<0.05$ (tested for using Students paired t test) is represented by the following characters:
§ monocytes vs. control Mø,
† cycloheximide treated non-apoptotic vs. apoptotic Mø,
* control Mø vs. apoptotic Mø,
# control Mø vs. cycloheximide treated non-apoptotic Mø,
¶ Non apoptotic vs. apoptotic U937 cells.
The mean results for each receptor are also shown. Note that antibodies C032, C033 and C054 all have a significant increase in binding on apoptotic U937 cells but apoptotic U937 cells also bind BOB 78 (which binds an intracellular antigen).

5. 5 Appendix 3

Cytokine Receptor Expression on Human Polymorphonuclear Leukocytes and the Changes Associated with Apoptosis. Summary of Changes with Apoptosis in Neutrophils, Macrophages and U937 Cells.

CD25 (IL-2 receptor α)

Panel Code	PMN	Apop PMN	sig.	Relative change with Apoptosis PMN	MØ	U937
C003	1.16	1.17	n/s	⇔	⇔	⇔
C010	1.88	2.23	n/s	⇔	ND	⇓

CDw 116 (GM-CSF receptor)

Panel Code	PMN	Apop PMN	sig.	Relative change with Apoptosis PMN	MØ	U937
C007	3.23	2.62	n/s	⇔	⇓	⇓

CD117 (SCF receptor or *c-kit*)

Panel Code	PMN	Apop PMN	sig.	Relative change with Apoptosis PMN	MØ	U937
C054	1.08	1.14	n/s	⇔	ND	⇑

CD120a (TNFR type 1 or p55)

Panel Code	PMN	Apop PMN	sig.	Relative change with Apoptosis PMN	MØ	U937
C025	1.62	1.25	n/s(p0.1)	(⇓)	⇓	⇓
C042	1.32	1.06	n/s(p0.07)	(⇓)	⇓	⇓

CD120b (TNFR type 2 or p75)

Panel Code	PMN	Apop PMN	sig.	Relative change with Apoptosis PMN	MØ	U937
C006	1.99	1.39	n/s	⇔	⇓	⇔
C029	5.57	1.16	<0.05	⇓	⇓	⇓
C043	2.22	1.03	<0.05	⇓	⇓	⇓

CDw 121a (IL-1R type 1)

Panel Code	PMN	Apop PMN	sig.	Relative change with Apoptosis PMN	MØ	U937
C001	3.31	2.58	n/s	⇔	ND	⇓

CDw 121b (IL-1R type 2)

Panel Code	PMN	Apop PMN	sig.	Relative change with Apoptosis PMN	MØ	U937
C002	5.12	3.78	n/s	⇔	⇓	⇓

CD122 (IL-2 receptor β)				Relative change with Apoptosis		
Panel Code	PMN	Apop PMN	sig.	PMN	MØ	U937
C048	1.48	2.71	n/s	⇔	⇓	⇓

CDw 124 (IL-4R)				Relative change with Apoptosis		
Panel Code	PMN	Apop PMN	sig.	PMN	MØ	U937
C004	1.32	1.44	n/s	⇔	⇓	⇔

CD126 (IL-6R)				Relative change with Apoptosis		
Panel Code	PMN	Apop PMN	sig.	PMN	MØ	U937
C020	4.57	2.01	n/s	⇔	⇔	⇓
C059	1.45	1.06	n/s	⇔	⇓	⇔

CDw 128 (IL-8R)				Relative change with Apoptosis		
Panel Code	PMN	Apop PMN	sig.	PMN	MØ	U937
C069	1.00	1.04	n/s	⇔	ND	⇔
C070	1.27	1.36	n/s	⇔	ND	⇔

CD95 (Anti-Fas)				Relative change with Apoptosis		
Panel Code	PMN	Apop PMN	sig.	PMN	MØ	U937
C071	2.35	3.64	n/s	⇔	⇔	⇔

CDw 84 (unknown function)				Relative change with Apoptosis		
Panel Code	PMN	Apop PMN	sig.	PMN	MØ	U937
C056	1.10	1.06	n/s	⇔	⇓	⇔

Unclustered at the workshop				Relative change with Apoptosis		
Panel Code	PMN	Apop PMN	sig.	PMN	MØ	U937
C023	1.05	1.07	n/s	⇔	⇔	⇓
C033	1.23	1.28	n/s	⇔	ND	⇑
C077	12.3	7.4	n/s	⇔	⇔	⇑
C078	1.35	1.15	n/s	⇔	⇔	⇓

Controls				Relative change with Apoptosis		
	PMN	Apop PMN	sig.	PMN	MØ	U937
anti-CD16	105.2	1.21	<0.05	⇓	ND	ND
Bob 78	1.28	1.20	n/s	⇔	ND	⇑

Legend for Table 5. 3
Results of dual colour immunofluorescence for PMN aged in culture, mean 62% apoptosis (range 53 - 70%, n = 3). Non-apoptotic PMN express high levels of CD16 whilst apoptotic PMN lose this; using a FITC conjugated anti-CD16 mAb non-apoptotic cells with high FITC fluorescence counts (PMN) could be distinguished from the apoptotic cells with low FITC fluorescence (Apop PMN). Gating separately on these two sub-populations the binding of mAb from the cytokine receptor panel could be determined using a PE conjugated second layer. Results are expressed as relative mean fluorescence, dividing the log fluorescent counts of test mAb by that of a PE conjugated non-binding control (MOPC-21C), no specific binding is indicated by 1.00. Relative binding for PMN and Apop PMN were compared using Students paired t test and significance shown in the column labelled Sig. (n/s = not significant).

On the right side of the table is a summary for each antibody of the change associated with apoptosis for neutrophils (PMN), macrophages (MØ) and U937 cells (U937) where:

⇔ = no change in expression between non-apoptotic and apoptotic cells,

⇓ = reduced expression with apoptosis,

⇑ = increased expression with apoptosis,

ND = not done.

(Values in brackets indicate a trend only.)

Chapter 6

THE ROLE OF FREE RADICALS AND ONCOGENES IN MONOCYTE AND MACROPHAGE APOPTOSIS.

6. 1 INTRODUCTION.

In this chapter I aimed to investigate potential sources of the differential sensitivity to the induction of apoptosis noted in Chapter 3 between monocytes and Mø. In the light of the roles of oncogenes in the regulation of apoptosis and the relative resistance of Mø to the induction of apoptosis, I looked the level of expression of specific oncogene products in monocytes and Mø. Bcl-2 (Hockenbery, 1993) and ABL (Bedi, 1994) have been shown to be important in preventing and c-Myc (Amanti, 1993) in promoting apoptosis. Although monocytes have been reported to weakly express *bcl*-2, changes in expression during monocyte differentiation have not been investigated (Iwai, 1994). Up-regulation of *bcl-2* expression with Mø maturation could explain the lower levels of constitutive Mø apoptosis and their resistance to the induction of apoptosis by factors which cause monocyte apoptosis. As Bcl-2 has been proposed to protect cells against free radical induced apoptosis and mononuclear phagocytes are powerful sources of ROI, I also investigated whether free radicals are important in inducing phagocyte apoptosis and whether there was a role for endogenous antioxidants in preventing this. Since monocytes and Mø have a powerful array of endogenous antioxidants, it is attractive to speculate that their intrinsic resistance to the induction of apoptosis may reside, at least partially, in their ability to cope with oxidative stress, either through the action of Bcl-2 or their endogenous antioxidant capacity.

I determined the levels of expression of Bcl-2, c-Myc and BCR-ABL in monocytes and the changes in expression with maturation into Mø using antibody probes in permeabilised cells. As positive controls I employed

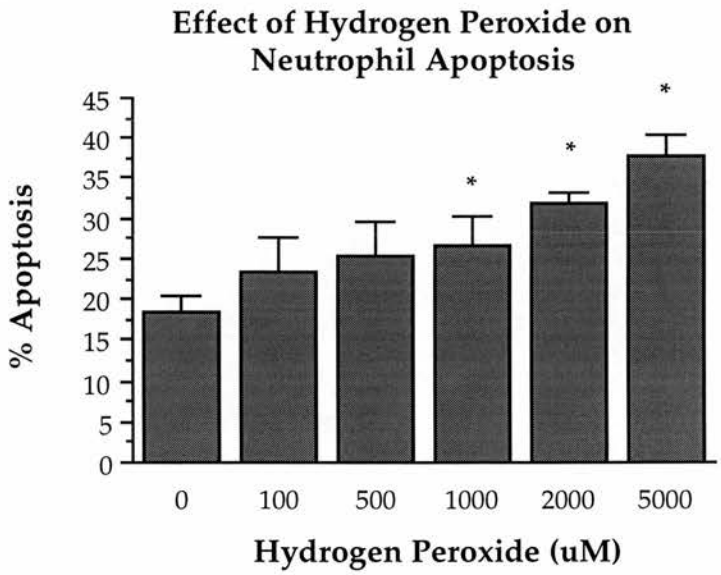
an mAb against p8,14 which are two associated calcium binding proteins specific to myeloid cells where they are abundant in the cytoplasm (Edgeworth, 1989 and 1991) and also a mAb against MPO. I also investigated whether ROI could induce phagocyte apoptosis by the addition of hydrogen peroxide (H_2O_2) to cultured cells and the effect of different antioxidants on the action of H_2O_2.

6. 2 RESULTS:

6. 2. 1 Hydrogen Peroxide Promotes Neutrophil Apoptosis in a Dose Dependent Manner.

Due to their availability, initial studies examined PMN to establish the dose response for an ROI and antioxidant; investigations were then extended to monocytes and Mø. H_2O_2 (1 - 5 mM) significantly promoted PMN apoptosis as shown in **Figure 6. 1**, without affecting viability.

Figure 6. 1



Legend for Figure 6. 1

Hydrogen peroxide added to fresh PMN cultured in 2% autologous serum in 8 well slides under standard conditions. After 8 hours cells

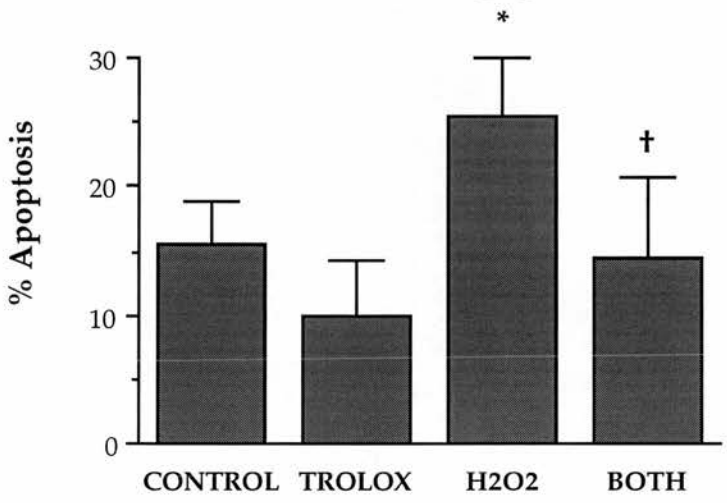
were resuspended and cytospins made. Apoptosis was assessed morphologically, 400 cells were counted per slide and duplicate slides were made for each treatment. Results are Mean ± SE, n = 9.

6. 2. 2 Antioxidants Block the Apoptosis Promoting Action of Hydrogen Peroxide.

Two different antioxidants were initially tested; the soluble vitamin E analogue Trolox and the endogenous antioxidant GSH. Trolox significantly inhibited the apoptosis promoting effect of H_20_2 on PMN.

Figure 6. 2

Effect of Trolox on Hydrogen Peroxide Induced PMN Apoptosis.



Legend for Figure 6. 2

PMN were cultured in 8 well slides in 2% autologous serum (CONTROL), or with the addition of Trolox 10 mM (TROLOX), 1 mM H_20_2 (H_2O_2) or both added simultaneously (BOTH). Apoptosis was assessed morphologically after 8 hours. * Significant increase in apoptosis above control level ($p<0.005$). † Significant reduction in apoptosis from H_2O_2 levels ($p<0.0005$, n = 8). Trolox alone tended to reduce baseline apoptosis, but was not significant, ($p = 0.23$, n = 8).

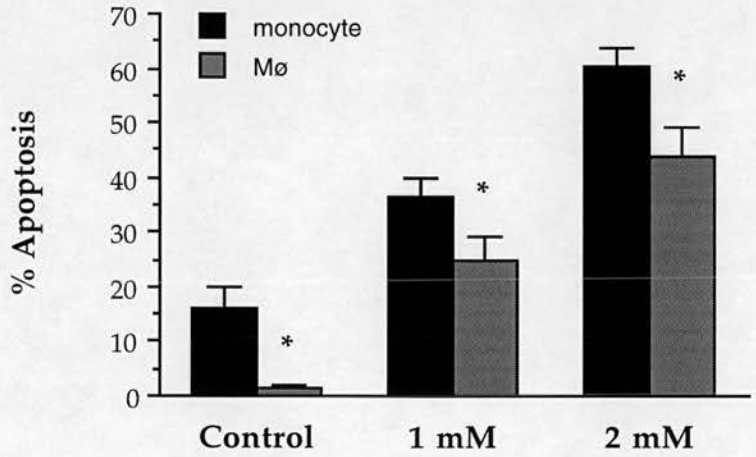
GSH also appeared to inhibit the apoptosis promoting effect of H_2O_2, with the apoptosis levels of control, H_2O_2 (1 mM) and H_2O_2 with 2 mM GSH being 16.4 ± 4%, 20.6 ± 2.1% and 13.9 ± 4.3% respectively, however due to the small numbers used (n = 3) this did not reach significance. Neither Trolox or GSH affected cell recovery or trypan blue exclusion rates.

6. 2. 3 Hydrogen Peroxide Promotes Both Monocyte and Macrophage Apoptosis.

Having established that H_2O_2 augmented PMN apoptosis, this system was tested on monocytes and Mø, the results are shown in **Figure 6. 3**.

Figure 6. 3

Hydrogen Peroxide Induced Monocyte and Macrophage Apoptosis.



Legend for Figure 6. 3

Monocytes and corresponding Mø (matured *in vitro* for 5 days), cultured in 8 well slides in 2% autologous serum for 20 hours (Control) or with the addition of H_2O_2 1 mM or 2 mM. Apoptosis was counted morphologically. The addition of 1 mM H_2O_2 caused a significant increase in both monocyte and Mø apoptosis ($p<0.01$) over

Control and 2 mM H_2O_2 caused a further significant increase in both monocyte and Mø apoptosis ($p<0.01$) over the 1 mM H_2O_2 levels.

* Mø apoptosis levels were significantly lower than for monocytes. Results expressed as mean ± SE, n = 5.

Monocytes were cultured in 2% and 10% serum, the reduction in serum significantly increased apoptosis rates in the presence of H_2O_2 as shown.

Table 6. 1

Hydrogen Peroxide Causes Apoptosis in Human Monocytes Which is Partially Prevented by Serum.

Treatment	Serum %	% Monocyte Apoptosis	p value (10% vs. 2%)	n
Control	10%	13.0 ± 2.7		6
	2%	15.8 ±4.1	n/s	6
1 mM H_2O_2	10%	17.6 ±4.1		5
	2%	36.6 ±3.1	<0.01	5
2 mM H_2O_2	10%	30.6 ±3.1		5
	2%	60.4 ±3.2	<0.01	5

Legend for Table 6. 1

Monocytes were cultured in 10% or 2% autologous serum for 20 hours on 8 well slides, H_2O_2 (to a final concentration of 1 or 2 mM) was added only at the start of the experiment. Apoptosis was

determined morphologically by acridine orange fluorescent microscopy, 1000 cells being counted per treatment well. Results expressed as mean ± SE. Comparison was made between monocytes cultured in the presence of 10 or 2% serum for Control, 1 mM H_2O_2 and 2 mM H_2O_2 using Student's paired t test. There was no significant difference in the levels of apoptosis for Control cells whilst for both 1 and 2 mM H_2O_2 there was significantly greater levels of apoptosis in the presence of 2% serum than that in 10% serum. The number of experiments per group is shown in table (n). DNA electrophoresis again confirmed that apoptosis was associated with DNA fragmentation and that H_2O_2 augmented this.

As with serum withdrawal alone, "ghost" cells were seen in monocyte cultures in the presence of H_2O_2. They were more abundant when the percentage of apoptosis was higher but were seldom more than 10% of cells, again they were rare in Mø cultures. In a single experiment, 1 mM H_2O_2 was added to alveolar Mø in an 8 well slide for 12 hours, apoptosis was increased from 5.6% in the 3 control wells to 12.4% in the 3 well exposed to H_2O_2 suggesting that these cells too are susceptible to H_2O_2 induced cell death.

6. 2. 4 Antioxidant Capacity of Donor's Serum Shows Little Variability.

To determine whether individual differences in serum antioxidant capacity could influence the results, the serum Trolox equivalent antioxidant capacity (TEAC) for a number of donors serum was determined as described in section 2. 7. 3. 4. The mean TEAC value was 1.12 (range 1.08 to 1.19, n = 5) which was well within the published

normal range for human serum of 1.0 to 1.5 (Miller, 1993). The tight range of these results suggests that variations in the antioxidant capacity of serum was not a significant factor in the results.

6. 2. 5 Oncogene Expression During Monocyte Differentiation.

The expression of Bcl-2, c-Myc and c-Abl were determined in permeabilized monocytes from 7 donors. Monocytes from the same donors were matured into Mø and the relative changes in expression of these oncogene products were followed using indirect immunofluorescence. Antibodies against the enzyme MPO and the cytoplasmic protein p8,14 were used as the positive controls. HL-60 cells were used as a positive control for the anti-Bcl-2 mAb and fibroblasts for the anti-c-Myc mAb. The relative mean fluorescence of anti-Bcl-2 mAb on HL60 cells was 10.9 ± 2.6 and that of the anti-c-Myc mAb on fibroblasts was 23.3 ± 7.8 (S. Hannah, personal communication). Monocytes were shown to express low levels of Bcl-2 but despite the increased resistance to the induction of apoptosis shown by Mø, the levels of Bcl-2 expression fell significantly during the maturation of monocytes into Mø as shown in **Table 6. 2.**

Table 6. 2

Oncogene Expression in Human Monocytes and Macrophages.

GENE PRODUCT	MONOCYTES	MACROPHAGES	p Value
Bcl-2	3.47 ± 0.7	1.8 ±0.3	< 0.05
c-Myc	21.8 ± 8	7.3 ± 1.5	n/s
c-Abl	1.0 ± 0.2	1.4 ± 0.5	n/s
Myeloperoxidase	67 ± 18	7.3 ± 2.6	< 0.05
p8,14	78 ± 18	45 ± 12	n/s

Legend for Table 6. 2

Expression of the gene products of the oncogenes *bcl-2, c-myc* and *c-abl* in monocytes and corresponding Mø populations, shown by mAb to permeabilized cells (polyclonal antibody for c-Abl). MPO and p8,14 included as positive controls, all binding expressed as relative mean fluorescence ± SE related to that of the negative control MOPC-21C. No expression gave a value of 1.0 - the same as the negative control. For c-Abl, polyclonal sheep serum was used as control. Monocyte and Mø populations were >95% pure. In the cases where there was positive expression of gene product, greater than 90% of cells were positive. Students paired t test used to test for significance, n = 7.

Although there was an overall fall in the mean expression of c-Myc from monocyte to Mø this masked the fact that expression was markedly reduced in three donors whilst in the remaining four slightly increased expression was observed (**Figure 6. 4a**). This differed from the results for Bcl-2 where there was a uniform fall in the levels of gene product during maturation (**Figure 6. 4b**). The reasons for the variable pattern of expression of c-Myc are not clear, the same preparative method was used for all cells and neither sex nor age could be used to distinguish donors with a fall or rise in c-Myc. These studies were all performed over a two month period and the mAb used all came from the same stock. Altering the time that the cells were exposed to the permeabilization solution did not affect the results (data not shown). It is of interest that those donors with high c-Myc expression also had higher Bcl-2 expression.

Figure 6. 4a

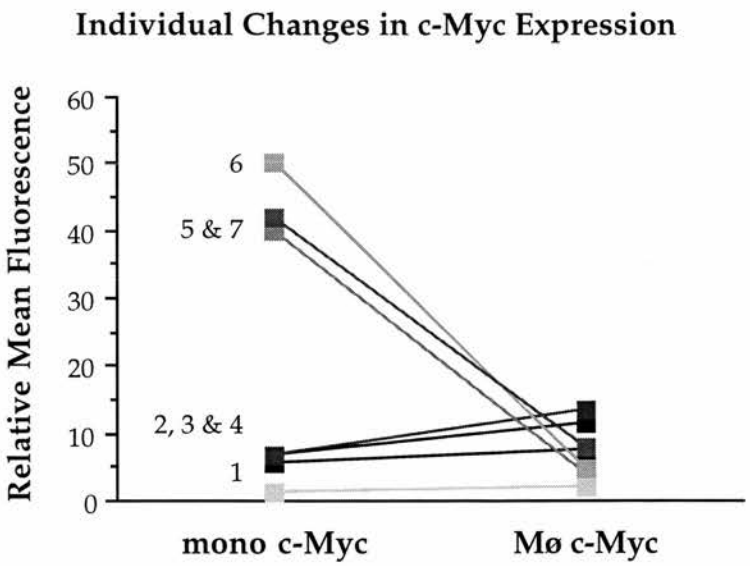
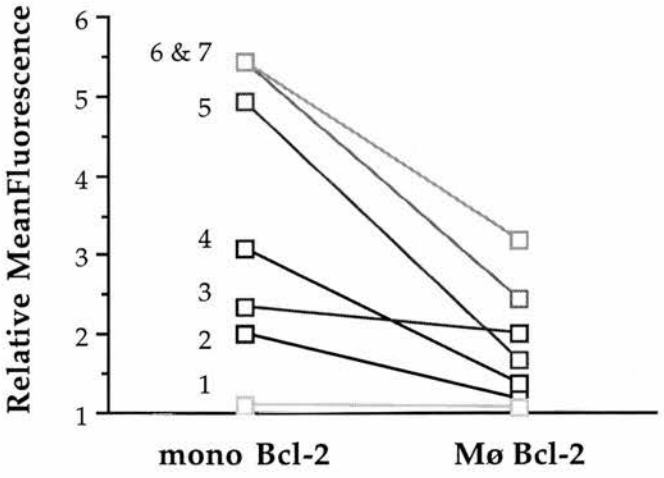


Figure 6. 4b

Individual Changes in Bcl-2 Expression



Legend for Figure 6. 4

Changes in expression of Bcl-2 and c-Myc during monocyte to Mø maturation for each donor (numbered 1 to 7, the same numbers are used for c-Myc and Bcl-2 expression). Results are shown as relative mean fluorescence for monocytes and the corresponding values for the same donors Mø examined 5 days later after *in vitro* maturation

Looking at the ratio of Bcl-2 to c-Myc for monocytes and Mø a more consistent pattern emerged with a ratio of 0.26 (range 0.11 to 0.5) for monocytes and 0.32 (0.12 to 0.68) for Mø, indicating a persistent excess of c-Myc. The results for c-Abl were somewhat inconclusive as the binding of the control sheep serum was strikingly positive on monocytes and Mø. The polyclonal nature of this control may thus have masked any specific positive binding of the anti-Abl antibody, mAb against c-Abl were not available at the time.

6. 3 DISCUSSION.

These data demonstrate that the H_2O_2 significantly increases the rates of apoptosis for PMN, monocytes and Mø. This is the only "physiological" agent I have so far tested that does promote apoptosis in the monocyte/Mø system although it was being used at pharmacological concentrations. For PMN, the antioxidant Trolox could prevent the pro-apoptotic effect of H_2O_2 (see **Figure 6. 2**), a protective effect which may be shared by other antioxidants such as GSH. It is interesting to note that Trolox also tended to decrease the baseline rate of apoptosis in PMN which would be consistent with a role for oxidative stress in the normal constitutive induction of apoptosis in these cells in culture. In these studies of the effect of exogenous antioxidants, the soluble nature of Trolox is important. Abello *et al.* demonstrated that only soluble antioxidants (dimethyl sulfoxide and the iron chelator - o-phenanthroline) were able to block endothelial cell apoptosis whilst cell impermeable scavengers (superoxide dismutase and catalase) were without effect (Abello, 1994). There is close agreement between the concentrations of Trolox (10 mM) required in my work to protect cells against H_2O_2 induced apoptosis and that used by Forrest *et al.* (Trolox 10 mM) to protect thymocytes from H_2O_2 induced damage (Forrest, 1994).

Serum was shown to have a significant protective effect for monocytes exposed to H_2O_2. The antioxidant capacity of serum is well recognized and it is probable that it is though these antioxidant mechanisms that serum protects monocytes from ROI induced apoptosis. To control for the possibility that serum from different donors varied in antioxidant capacity, these levels were determined using the TEAC assay. This

demonstrated that all the donors had a remarkably similar antioxidant capacity which fell entirely within the normal range (Miller, 1993).

H_2O_2 has been shown to induce apoptosis in a number of other cells but at significantly lower concentrations than needed here. HL60 cells, cultured in 10% serum, underwent apoptosis in response to only 15 μM H_2O_2, with higher concentrations (400 μM) causing necrosis (Lennon, 1991) and as little as 25 nM H_2O_2 could result in lymphocytes loss (Greenspan, 1994). However in my hands PMN, monocytes and Mø all required at least 1 mM H_2O_2 to induce significant apoptosis without evidence of cell necrosis, the concentration of the H_2O_2 used was checked prior to each experiment as described in section 2. 7. 3. 3. The concentrations of H_2O_2 required to cause Mø cell death in this work agrees with results obtained by Schraufstatter *et al.* who showed that 10 mM H_2O_2 was required for rabbit alveolar Mø death (Schraufstatter, 1987). The explanation for the difference in H_2O_2 requirement between phagocytes and other cells may well lie in the endogenous antioxidant capacity of phagocytes. The susceptibility of cells to ROI induced damage is inversely correlated to their catalase content (Schraufstatter, 1987), furthermore adult T cell leukemia cells are protected from ROI mediated cell death by increased concentrations of glutathione (Kohno, 1996). Activated PMN have been shown to produce large amounts of superoxide and H_2O_2 (Weening, 1975; Forman, 1986). Measurements of H_2O_2 and superoxide released into the surrounding medium show production rates of 1000 μM per 10^{10} PMN per hour (Joke, 1975). Quantification of the intracellular generation of oxygen metabolites has not been possible but chemiluminescence assays indicate that this is in fact greater than the measured extracellular production (Briheim, 1989). Thus the cell requires considerable

protection against its own armoury of ROI, hence the redundancy in antioxidant mechanisms as discussed in section 1. 4. 2. 4.

This chapter also documents changes in oncogene expression during monocyte to Mø maturation. The two positive controls (MPO and p8,14) confirmed both Mø maturation and effective cell permeabilization. MPO is known to decline during the maturation of monocyte into Mø (Savill, 1989a) and this was observed. p8,14 are infact two non-covalently bound calcium binding proteins that fractionate together. The p8,14 calcium binding proteins are specific to myeloid cells, representing 45% of PMN cytosolic protein but considerably less in monocytes (Edgeworth, 1989 and 1991) and provide a useful cytoplasmic antigen positive control that remained strongly positive in both monocytes and Mø.

Surprisingly, the expression of the oncogene product Bcl-2 was very low in monocytes and despite the increased resistance of the Mø to the induction of apoptosis, the expression of Bcl-2 fell significantly with maturation into the Mø. These findings also corroborate and extend the work of Delia *et al.* who demonstrated that in human bone marrow preparations, the myeloid precursors myeloblasts, promyelocytes and myelocytes stain positive for Bcl-2 whereas monocytes were negative (Delia, 1992). The PMN has also been shown to not express Bcl-2 (Delia, 1992; Hannah, 1995). Iwai *et al.* investigated the expression of Fas antigen and Bcl-2 on human peripheral blood lymphocytes, monocytes and PMN. Lymphocytes expressed high levels of Bcl-2 whilst monocytes had only very weak Bcl-2 expression and PMN did not express it at all, they correlated this with susceptibility to undergo apoptosis (Iwai, 1994). In

view of the lack of expression of Bcl-2 by Mø demonstrated in this work, resistance to apoptosis by these cells must lie in other mechanisms.

As noted in the introduction the *bcl-2* oncogene product is part of a large family of molecules including Bcl-x-$_L$ and Bak which are able to inhibit apoptosis (Reed, 1994; Kiefer, 1995). Due to the absence of suitable antibodies at the time of this study I was unable to look for these gene products, hence the possibility remains that despite the absence of Bcl-2 expression, resistance to apoptosis in the Mø could still be conferred by a Bcl-2 family member. Reed's group have documented the *in vivo* expression of Bax and Bcl-X (Krajewski, 1994a and 1994b). They were unable to clearly distinguish between Bcl-x-$_L$, (an inhibitor of apoptosis) and Bcl-x-$_S$ which like Bax promotes apoptosis. Although Mø are long-lived cells, they did not single out Mø as notable for Bcl-x-$_L$ expression, indeed Bcl-x-$_L$ was not expressed on glial cells and rarely in normal lymph nodes. In the bone marrow they noted some myeloid precursors expressed Bcl-X but commented that expression may well be lost with terminal differentiation. Furthermore Benito *et al.* have recently demonstrated that differentiation of erythroid human leukemia cell lines resulted in loss of Bcl-x-$_L$ mRNA and the onset of apoptosis (Benito, 1996). Thus differentiation of monocytes into Mø may not be associated with significant expression of Bcl-x-$_L$ and although other members of the Bcl-2 family may be involved in Mø resistance to apoptosis, initial studies do not suggest that Mø are strongly Bcl-x-$_L$ positive and Bax negative.

c-Myc levels are related to the stage of cell cycle in proliferating cells and are known to decline when myeloid cell lines are induced to differentiate, with a switch from Myc:Max to Mad:Max heterodimers accompanying

U937 differentiation (Marcu, 1992; Ayer, 1993). However these reports are not on primary cultures of human cells, hence the changes in expression of c-Myc with maturation from monocytes into Mø are of interest. The decline in relative mean fluorescence from 21.8 ± 8 to 7.3 ± 1.5 (**Table 6. 2**) fits with that expected from studies using cell lines but masks a wide individual variation with 4 of the 7 subjects actually showing a small rise in expression with differentiation (Figure 6. 4a). Thus it would seem unlikely that changes in c-Myc expression account for the greater resistance of Mø to the induction of apoptosis than the monocyte.

The *BCR-ABL* gene has been shown to inhibit apoptosis in myeloid progenitor cells, inhibition of *BCR-ABL* gene by anti-sense oligonucleotides reverses this suppression of apoptosis. This is presumed to underlie the growth advantage of Philadelphia cells in chronic myeloid leukaemia (Bedi, 1994). Furthermore over-expresion of *abl*, using a temperature sensitive mutant, prevents apoptosis in HL-60 cells (Martin, 1995). Recently it has also been suggested that the *myc* gene cooperates with *BCR-ABL* during cell transformation (Skorski, 1995). By the assay used here the detected levels of the c-Abl oncogene product were extremely low and no significant difference between monocytes and Mø was observed. This result needs to be interpreted with caution as the anti-Abl antibody used was a polyclonal and binding of the polyclonal sheep immunoglobulin control was much greater than that of MOPC-21C thus maturational changes in c-Abl may have been missed. In summary these data show that there is no significant change in the ratio of Bcl-2 to c-Myc, nor in the levels of BCR-ABL during the Mø maturation process. This suggests that increased Mø resistance to apoptosis is not conferred by changes in these oncogene products.

Chapter 7

THE IN VIVO FATE OF THE INFLAMMATORY MACROPHAGE DURING THE RESOLUTION OF INFLAMMATION.

7.1 INTRODUCTION.

Although the inflammatory Mø plays a central role in the inflammatory process its fate during the resolution of inflammation is largely unknown (Adams, 1992). Resolution of acute inflammation is characterized by the clearance of extravasated PMN and Mø and the return of normal tissue architecture. However, in a number of disease processes inflammation does not resolve, rather it persists and is often associated with fibrosis and loss of organ function (Adams, 1992), as exemplified by chronic bronchitis, emphysema (Khalil, 1989), glomerulonephritis (Baker, 1994) or rheumatoid arthritis (Koch, 1991 and 1994). The mechanisms underlying the development of chronic inflammation are poorly understood but much attention has been focused on the role of the inflammatory Mø. The Mø can damage tissue by release of histotoxic enzymes, pro-inflammatory and pro-fibrogenic cytokines or presentation of antigen to T cells (Nathan, 1987; Khalil, 1989; Adams, 1992) and although Mø are able to debride tissue and promote wound healing (Riches, 1988), continued Mø accumulation is also a hallmark of chronic inflammation. Thus, definition of the mechanisms controlling not only the influx but also the persistence and removal of inflammatory Mø are important to our understanding of the pathogenesis of both acute and chronic inflammation.

The origin and influx of monocytes have been well documented (Ebert, 1939; Rosen, 1990; van Furth, 1992), whilst the fate of the inflammatory Mø has received comparatively little attention. Inflammatory Mø are known to be derived from circulating monocytes which migrate into acute inflammatory reactions, mixing with the resident Mø population

(van Furth, 1973). The kinetics of resident tissue Mø in the non-inflamed steady state have also been the subject of investigation (Rosser, 1970; van Furth, 1992), but resident and inflammatory Mø differ in cell physiology and activation status (Daems, 1973; Haskill, 1985; Melnicoff, 1988a; van Furth, 1989) and may differ in ontogeny (Volkman, 1976; Bouwens, 1986; Ginsel, 1993), hence the direct relevance of studies on the resident Mø to our understanding of the tissue kinetics of the inflammatory Mø is unclear.

Some insights into the cellular mechanisms underlying resolution of inflammation have been provided recently by studies relating to the fate of extravasated PMN during the resolution of inflammation. These cells have been shown to undergo apoptosis (Savill, 1989a) and subsequently are ingested by inflammatory Mø (Savill, 1989a; Grigg, 1991) utilizing a novel recognition mechanism (Savill, 1989b, 1990 and 1992) which fails to stimulate the release of pro-inflammatory mediators (Meagher, 1992). Thus, it has been suggested that this process may represent a non-phlogistic mechanism for the "silent" clearance of large numbers of PMN's and their potentially histotoxic contents during the resolution of acute inflammation (Haslett, 1994). Mø *in vitro* have now been shown to undergo apoptosis in response to a number of stimuli (Zychlinsky, 1992; Albina, 1993; Khelef, 1993; Papadimitriou, 1993; Sarih, 1993a and b). Hence it was entirely conceivable that, in a fashion analogous to the PMN or another phagocyte, the mesangial cell (Baker, 1994), the inflammatory Mø might undergo apoptosis in large numbers locally, and themselves become ingested by local phagocytes.

In order to address this question I have examined the kinetics of the inflammatory Mø using two independent labelling systems. Inflammatory Mø from H-$2^{k/d}$ donor mice were labelled with a red fluorescent dye and transferred into the peritoneal cavity of recipient H-2^{k} mice at the same stage of resolving inflammation as the donor mice. The use of fluorescent tracking dyes is now a well established technique allowing the stable labelling of cells in a non-toxic manner without apparently altering normal cell physiology (Melnicoff, 1988a and b; Soesatyo, 1993). Immunofluorescence analysis using a FITC anti-H-2^{d} antibody provided the second method of distinguishing donor or recipient cells and dual colour flow cytometry allowed identification of donor Mø that had been phagocytosed by recipient cells.

In the non-inflamed peritoneal cavity the half-life of resident Mø has been estimated to be 2 weeks or longer (Haskill, 1985; van Furth, 1989). Data presented in this chapter show that during the resolution process, adoptively transferred inflammatory Mø rapidly emigrate intact from the inflamed peritoneum specifically to the draining LN and that this emigration is complete by 96 hours, with little evidence of death or engulfment of Mø at the inflamed site. Furthermore the kinetics of inflammatory Mø emigration from the inflamed site are significantly faster than those of resident Mø from the non-inflamed peritoneum.

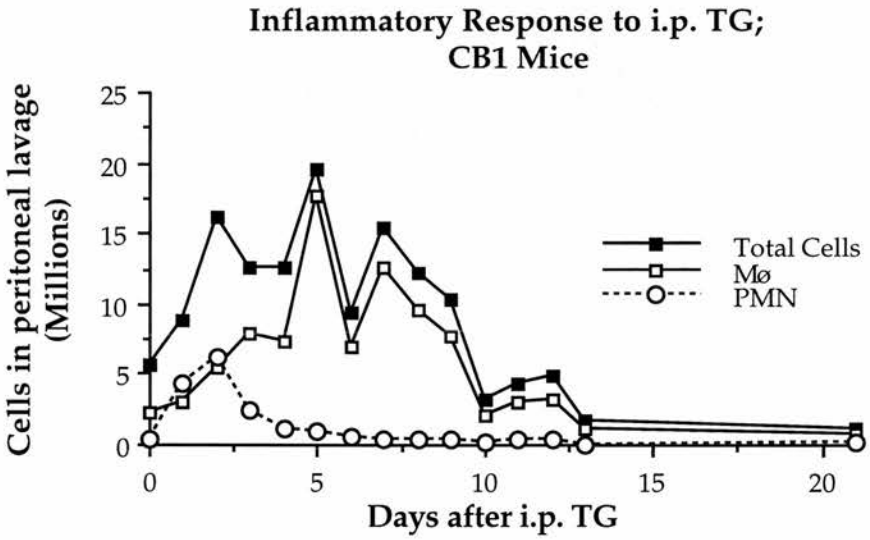
7. 2 RESULTS.

7. 2. 1 Time Course of Inflammatory Macrophage Response to Acute Inflammation.

Both CB1 and C3HF/KAM mice had less than 3×10^{6} resident Mø in the peritoneal cavity prior to the induction of inflammation. Approximately

40% of these cells were Mø, demonstrated by differential cell counts and by immunofluorescence, the bulk of the remaining cells being lymphocytes, in agreement with previous studies (Melnicoff, 1988b). TG induced a prompt PMN influx that declined after 2 days whilst for both CB1 and C3HF/KAM mice the Mø number ($19.6 \pm 1.8 \times 10^6$) and relative percentage (>85%) reached a peak on the 5th day after i.p. TG. As shown in **Figure 7. 1** the total number of cells and the number of Mø steadily declined from the fifth day after TG, thus this was chosen as the optimum day for cell transfer to track the kinetics of Mø during the resolution phase of inflammation.

Figure 7. 1



Legend for Figure 7. 1

Inflammatory cell response to i.p. TG for CB1 mice, showing the total number of leukocytes and the numbers of Mø and PMN in peritoneal lavageates with time. 2 mls TG was injected i.p. on day 0, cells were recovered using 5 mls ice cold PBS. Total cell number was estimated by haemocytometer, >400 cells being counted. Relative % of Mø and PMN were obtained from duplicate cytospin preparations (stained

with Dif Quick™ and for non-specific esterase) and by indirect immunofluorescence using the anti-CR3 mAb M1/70 for Mø and mAb 7/4 for PMN. Estimates of % Mø were equivalent for all three methods. The inflammatory response in C3HF/KAM mice was identical. Lymphocytes account for the remaining cells in the differential counts (not shown). Each point represent the mean for at least 4 mice; SD are not shown as they are all within 10% of the means . There was no significant difference in the kinetics of the Mø response to TG between CB1 and C3H mice.

A similar, although less pronounced, pattern was found using starch as the inflammatory agent, with $9.2 \pm 1.4 \times 10^6$ cells recovered on the 5th day after i.p. starch and again >85% were Mø. This declined significantly to $4.1 \pm 0.9 \times 10^6$ cells by day 10 ($p < 0.05$). Mø expressed CR3 (Beller, 1982) and F4/80 (Austyn, 1981; Hirsch, 1982; Hume, 1983), < 10% of cells expressed Ia, consistent with the observations of other investigators (Melnicoff, 1988b; Kimberly, 1992), (**Figure 2. 4** Chapter 2).

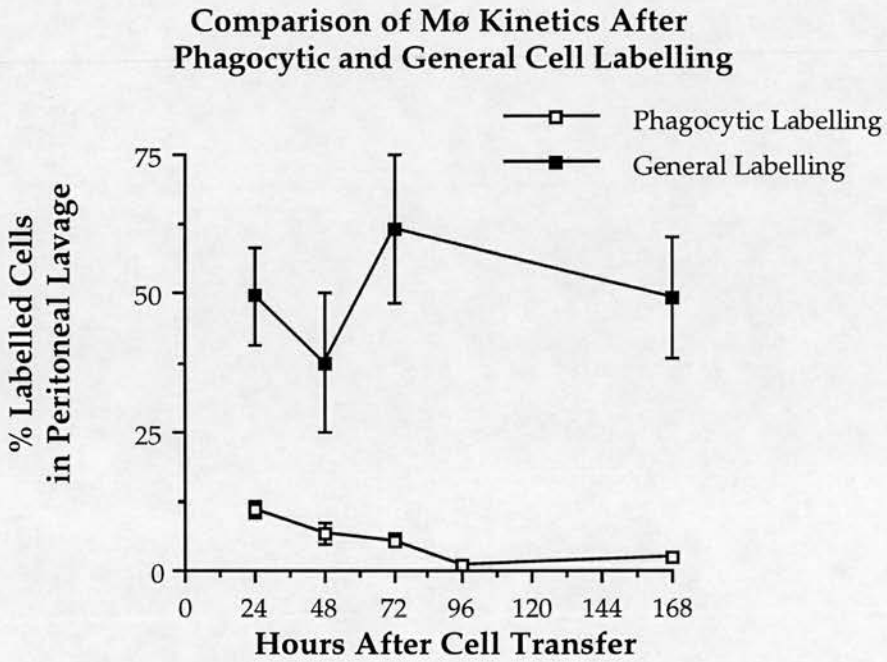
7. 2. 2 Adoptive Transfer of Donor Macrophages.

7. 2. 2. 1 *In Vitro* General Labelled Donor Macrophages.

At the outset it seemed appropriate to use a well defined method that labelled 100% of the donor cells, as could be achieved with *in vitro* PKH26-GL general cell labelling. However the cell viability after *in vitro* labelling became a source of concern. Altering a number of procedures including reducing the centrifuge speeds, number of washes and the concentration of dye all helped to improve viability however 10% of cells still were non viable at the time of transfer. Attempts to increase yields of viable cells by selective adherence to BHK matrix coated plates (see

section 2. 9. 5. 1) produced minimal improvements. **Figure 7. 2** shows the percentage of PKH26 labelled Mø recovered in peritoneal lavage after transfer of cells labelled by general *in vitro* and phagocytic *in vivo* labelling methods, the two are completely different in their kinetics. The *in vitro* general labelled Mø were handled in a manner similar to that of *in vivo* phagocytic labelled Mø that were formalin fixed (section 7. 2. 4).

Figure 7. 2



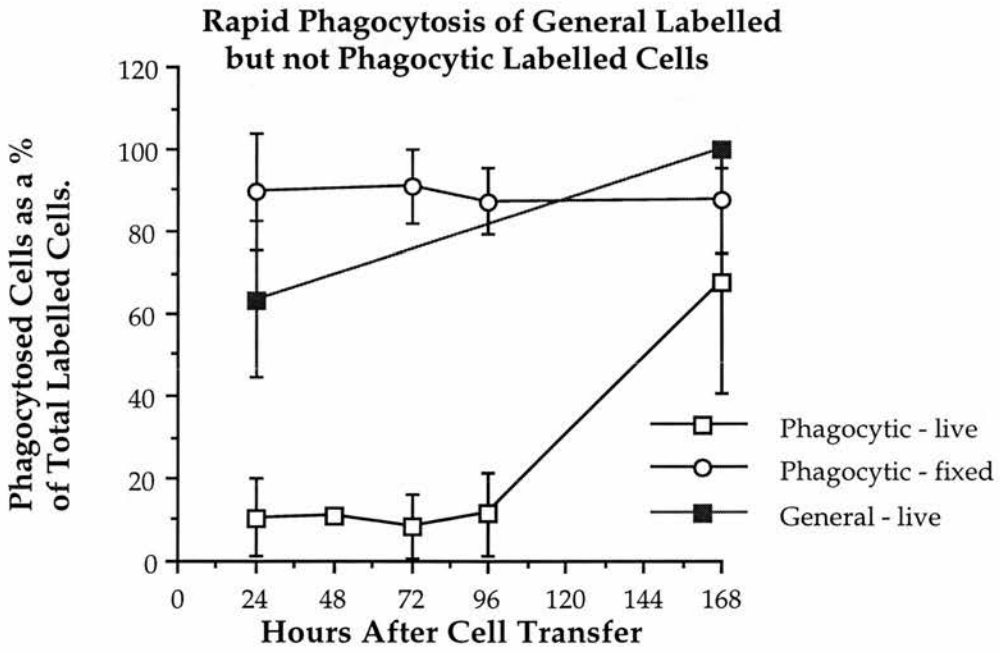
Legend for Figure 7. 2

Plot of recovery of donor CB1 inflammatory Mø from peritoneal cavities of C3HF/KAM mice after *in vitro* general cell labelling with PKH26-GL compared with that after *in vivo* phagocytic cell labelling with PKH26-PCL. 20 x 10^6 live donor *in vitro* labelled cells (100% labelled) and 30 x 10^6 live donor *in vivo* labelled cells (28 - 42% labelled) were adoptively transferred into recipient mice at the same stage of resolving inflammation. Each point represents the mean ± SE

for at least 8 mice receiving *in vivo* phagocytic labelled cells and 12 mice receiving *in vitro* general labelled cells.

Furthermore, it became apparent (**Figure 7. 3**) that there was considerable phagocytosis of *in vitro* labelled donor cells by recipient Mø after adoptive cell transfer, suggesting poor survival of these labelled cells, possibly as a result of damage during labelling. In view of these problems an *in vivo* phagocytic labelling system (PKH26-PCL; section 2. 9. 3. 1b and 7. 2. 2. 2) was tried. **Figure 7. 3** shows the degree of phagocytosis of *in vitro* and *in vivo* labelled donor Mø after adoptive cell transfer.

Figure 7. 3



Legend for Figure 7. 3

Plot of recovery from the peritoneum of PKH26 fluorescent labelled donor Mø that have been phagocytosed, expressed as a percentage of total number of fluorescent labelled cells recovered after adoptive transfer of: live donor *in vitro* general labelled cells (closed squares), live donor *in vivo* phagocytic labelled cells (open squares) or donor

in vivo phagocytic labelled cells that were formalin-fixed prior to adoptive transfer (open circles). Fixed *in vivo* labelled and live *in vitro* labelled cells were all rapidly phagocytosed. In contrast live *in vivo* labelled cells remained free in the peritoneum until one week after transfer when the few remaining cells were all phagocytosed. Each point represents the mean ± SE for at least 6 recipient mice for *in vivo* labelled cell transfer and 4 recipient mice for *in vitro* labelled cell transfer.

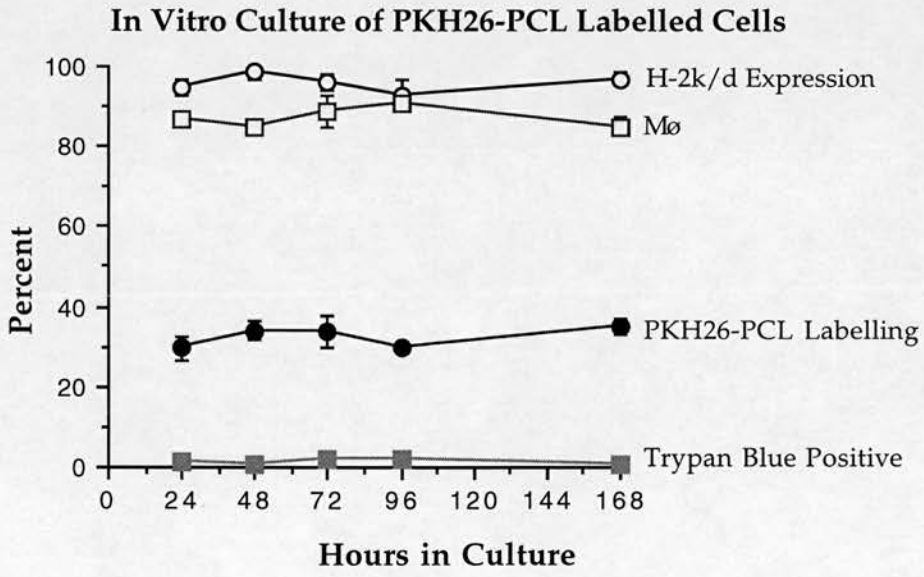
Thus *in vitro* labelling, although providing 100% labelling resulted in poor viabilities and rapid clearance of cells in a similar manner to that of fixed cells therefore *in vivo* labelling was adopted as the method of choice for the rest of this project despite the inability of this system to label all Mø.

7. 2. 2. 2 Adoptive Transfer of Live *In Vivo* Labelled Donor Macrophages.

In the absence of inflammation the phagocytic label PKH26-PCL has been reported to label all peritoneal Mø but Melnicoff *et al.* reported that inflammation significantly reduced the number of cells labelled (Melnicoff, 1990). I found that in the presence of inflammation, i.p. injection of 0.5 ml of fluorescent PHK26-PCL dye strongly labelled 1/3 of Mø (range 27 - 46.7%). This could be increased to 74% (61 - 82%) using 1 ml of dye and to over 80% with 2 mls of dye, although with 2 mls the viabilities began to fall. The percentage of cells labelled did not alter whether dye was left in situ for 1.5 or 18 hours prior to lavage. For the bulk of experiments 0.5 ml PKH26-PCL was used with a labelling time of 1.5 hours. This labelling distribution may reflect the rapid uptake of the

label and the small volume of the injectate, the supernatant from the lavage was not able to label further fresh cells *in vitro*, suggesting a rapid uptake of the dye locally, rather than the labelling of a subset of Mø. Indeed the characteristics of the labelled and unlabelled Mø were identical with regard to expression of CR3, F4/80, H-2^d and H-2^k as well as cell size, viability and ability to adhere to plastic and BHK matrix coated plates. Culture of *in vivo* labelled cells *in vitro* confirmed retention of PKH26-PCL and expression of H-$2^{k/d}$ over the course of the experiment, as shown in **Figure 7. 4**, with no difference in the viabilities of labelled or unlabelled cells; formalin fixation did not affect PKH26-PCL retention or H-$2^{k/d}$ expression (data not shown).

Figure 7. 4



Legend for Figure 7. 4

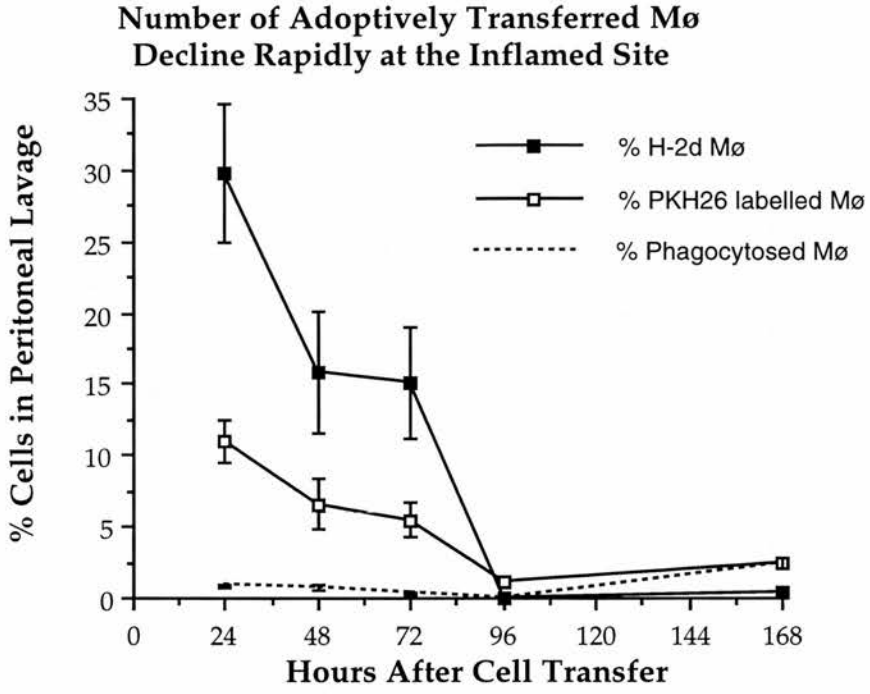
Cells (85.8 ± 1.9% Mø, 100% H-$2^{k/d}$, <1% non viable) were lavaged from peritoneal cavity of H-$2^{k/d}$ mice 5 days after i.p. injection of TG and 2 hours after i.p. injection of PKH26-PCL. These cells were cultured *in vitro* in Teflon foils for 7 days and examined daily for expression of H-$2^{k/d}$, PKH26-PCL, % Mø and viability, there was no

significant loss of PKH26-PCL fluorescence or H-$2^{k/d}$ expression during this time and cells remained fully viable as assessed by the ability to exclude the vital dye, trypan blue. (Mean ± SE, n = 3)

7. 2. 3 Adoptively Transferred Live Macrophage Numbers Decline Rapidly at the Inflamed Site.

To follow the resolution kinetics of inflammatory Mø, 30 x 10^6 CB1 cells (>95% viable and >85% Mø) all expressing H-$2^{k/d}$ and approximately one third of which were PKH26-PCL labelled, were transferred into the peritoneal cavities of C3HF/KAM (H-2^k) recipients at the same stage of resolving inflammation as the donor mice. **Figure 7. 5** shows the recovery of labelled cells from peritoneal lavage of recipient mice at different times after cell transfer. The percentage of H-$2^{k/d}$ cells and red fluorescent, PKH26-PCL labelled Mø declined proportionally over 96 hours effectively to zero. The ratio of PKH26-PCL labelled cells to H-$2^{k/d}$ cells remained approximately 1/3 over these 4 days, suggesting that the fate of labelled and unlabelled Mø was similar. There was little evidence of phagocytosis of the transferred Mø in the peritoneal cavity during the first 96 hours, with more than 90% of PKH26-PCL labelled Mø expressing donor H-$2^{k/d}$ rather than recipient H-2^k antigen. At one week (168 hours) after adoptive cell transfer the small percentage of red fluorescent cells remaining (3.7%), were almost all recipient Mø that had phagocytosed donor PKH26-PCL labelled cells. Despite the introduction of a large load of Mø, the number of Mø free in the peritoneal cavity fell steadily over the week, with a mean of 7 x 10^6 cells in the lavage 1 week after adoptive cell transfer, again consistent with resolving inflammation.

Figure 7. 5



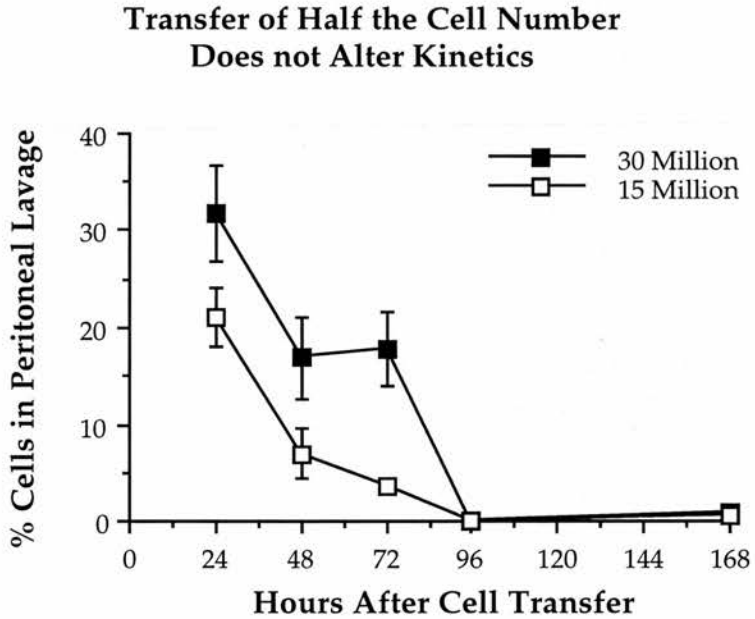
Legend for Figure 7. 5

Donor (H-$2^{k/d}$) Mø, PKH26-PCL labelled donor Mø and phagocytosed donor Mø expressed as a percentage of the total number of cells recovered from the peritoneal cavity of recipient (H-2^{k}) mice with time after adoptive transfer of 30 x 10^{6} live PKH26-PCL labelled donor cells. Each point represent the geometric mean of at least 6 recipient mice with 95% confidence intervals.

Using starch as the inflammatory challenge in an identical set of experiments, a similar pattern of clearance was seen. Donor Mø as a percentage of total Mø recovered in peritoneal lavage declined from 25 ± 5% 24 hours after semi-allogeneic cell transfer to 1.1 ± 1.9% 1 week after cell transfer ($p<0.05$, $n = 3$). Again there was little evidence of phagocytosis of donor Mø.

Transfer of half the number of donor cells did not affect the pattern of cell recovery, donor cells still taking 96 hours to completely disappear from peritoneal lavage (**Figure 7. 6**) suggesting that the rate of cell clearance was not dependant on the initial cell load.

Figure 7. 6



Legend for Figure 7. 6

Comparison of the decline in H-$2^{k/d}$ labelled Mø with time after the adoptive transfer of either 30 x 10^6 (closed squares) or 15 x 10^6 (open squares) live donor Mø into the peritoneal cavities of recipient mice. Each point represents the geometric mean and 95% confidence intervals for 6 mice for the 30 x 10^6 plot and 4 mice for the 15 x 10^6 plot.

The decline in PKH26-PCL labelled cells was also shown to be linear over the first 96 hours after the transfer of both 15 or 30 million cells, regression analysis showed that these slopes intersected at 1.5% PKH26-

PCL labelled cells (95% confidence intervals 0.8 - 2.5%) 96 hours after cell transfer. The H-$2^{k/d}$ slopes also intersected at 96 hours, their value at this time was zero, the 1.5% PKH26-PCL labelled cells at 96 hours could be accounted for by phagocytosis. The percent donor cells recovered 24 hours after transfer of 5, 10, 15, 30 and 50 x 10^6 cells show a steady increase as the donor cell load is increased, thus the recovery of donor cells at 24 hours as a percentage of the number initially transferred remains relatively constant, as shown in **Table 7. 1**.

Table 7. 1

Cell Recovery After 24 hours is Related to the Number of Donor Cells Adoptively Transferred.

Number of Cells Transferred	Number of Cells Recovered	Donor Cell Recovery as % of Cells Transferred	No. of Experiments
5 x 10^6	15.5 x 10^6	43%	2
10 x 10^6	19 ± 1.8 x 10^6	45%	3
15 x 10^6	22 ± 1.7x 10^6	32%	4
30 x 10^6	28 ± 2.1 x 10^6	28%	6
50 x 10^6	32 ± 1.8 x 10^6	34%	3

Legend for Table 7. 1

Increasing quantities of donor inflammatory Mø were transferred into recipient mice at the same stage of resolving acute inflammation. The total number of cells recovered 24 hours later and the number of donor cells recovered, expressed as a percentage of the initial number of donor cells transferred, were also determined.

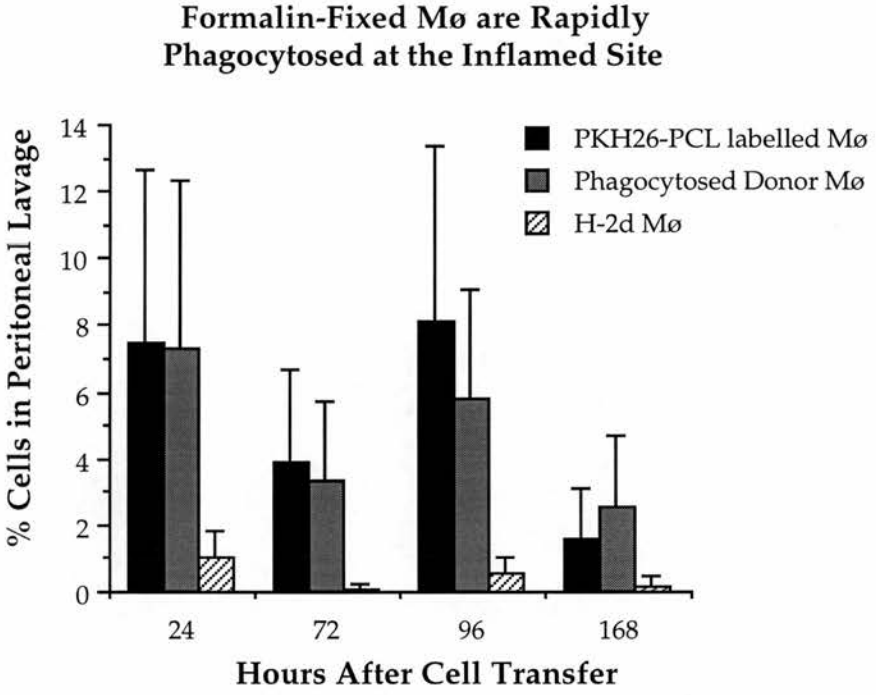
To determine whether the number of transferred cells recovered with time are appropriate to the original number transferred, the recovery of H-$2^{k/d}$ cells can be extrapolated back to the time of cell transfer (**Figure 7. 5**). This would suggest approximately 50% of the cells in the peritoneal cavity should be donor Mø immediately after adoptive cell transfer. Of the 30 x 10^6 cells transferred, 85% are Mø thus 25.5 x 10^6 will be H-$2^{k/d}$ Mø. The peritoneal cavity of the recipient mouse already contains approximately 20 x 10^6 free cells (**Figure 7. 1**), thus there are 25.5 x 10^6 H-$2^{k/d}$ Mø amongst a total of 50 x 10^6 cells - approximately 50%. A similar calculation pertains for PKH26-PCL labelled cells demonstrating that the percent cells recovered with time are appropriate to the number of cells transferred.

7. 2. 4 Adoptively Transferred Formalin Fixed Macrophages are Rapidly Phagocytosed at the Inflamed Site.

In order to address the question of whether the decline in live adoptively transferred cells required active emigration and to confirm that the system was capable of detecting phagocytosis of donor cells formalin fixed donor Mø were adoptively transferred in an identical set of experiments. The time course for recovery of these fixed CB1 cells from the peritoneal lavage samples after the transfer of 30x10^6 formalin fixed CB1 cells (>91% Mø) into C3HF/KAM recipients (n = 23 mice) is shown in **Figure 7. 7**. Virtually all the fixed cells were phagocytosed within 24 hours. In contrast less than 10% of live cells were phagocytosed over the whole 96 hours. Not only was the magnitude of phagocytic signal grossly different between live and fixed cells but the kinetics of live and fixed PKH26-PCL labelled cells also differed significantly by 96 hours. Plots of the percentage of live H-$2^{k/d}$ and PKH26-PCL labelled donor cells exhibited a

linear decline to 1.5% 96 hours after transfer whilst fixed cells exhibited no discernible trend; linearity was tested for by means of orthogonal (independent) polynomials as discussed in section 2. 11.

Figure 7. 7



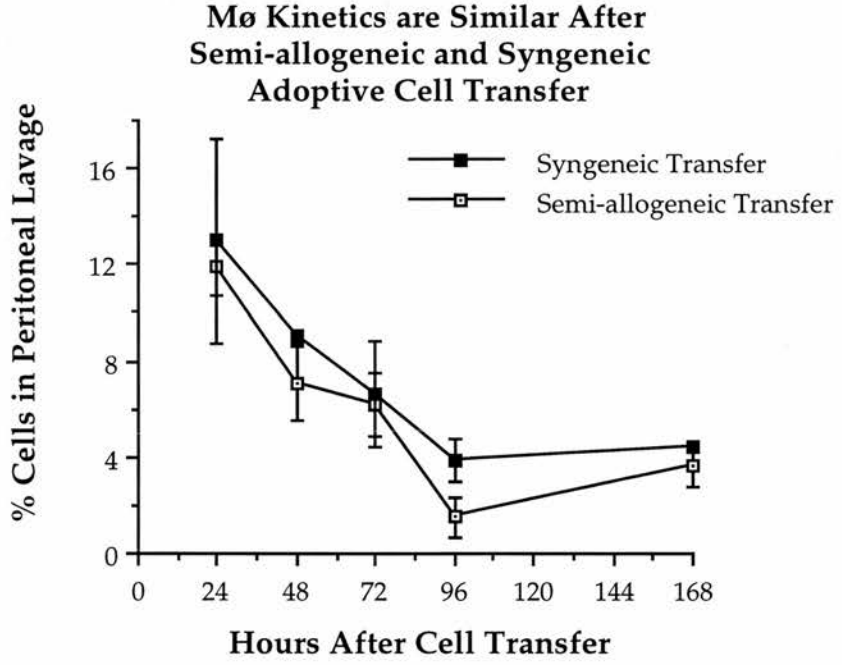
Legend for Figure 7. 7

Bar plot of the recovery of total PKH26-PCL labelled cells (black bars), recipient Mø that have phagocytosed labelled donor cells (stippled bars), and non-phagocytosed H-2^d expressing donor cells (hatched bars) expressed as a percentage of the total cells recovered, after the transfer of 30 x 10^6 formalin fixed donor cells into the peritoneal cavity of recipient mice. Each point represents the geometric mean for at least 6 recipient mice with 95% confidence intervals.

7. 2. 5 Semi-Allogeneic Macrophages Kinetics are the Same as Syngeneic Macrophages.

To investigate whether the presence of foreign antigen (H-2^d) on the adoptively transferred Mø caused recipient mice to handle these cells differently to their own, *in vivo* labelled cells from C3HF/KAM mice were transferred into C3HF/KAM recipients in an otherwise identical model of inflammation. The recovery of syngeneic PKH26-PCL labelled Mø in peritoneal lavage was compared with that of semi-allogeneic Mø, results are shown in **Figure 7. 8**.

Figure 7. 8



Legend for Figure 7. 8

Recovery of PKH26-PCL labelled donor Mø from peritoneal cavity of recipient mice with time after syngeneic (C3HF/KAM into C3HF/KAM - shown by closed squares), or semi-allogeneic (CB1 into C3HF/KAM - shown by open squares) adoptive cell transfer. Each

point represents the geometric mean of at least 4 recipient mice with 95% confidence intervals.

Kinetics of cells after semi-allogeneic transfer thus closely resemble that of syngeneic cells. Similar results were obtained for BALB/c as well as CB1 syngeneic transfers. The absence of any observed renewal of the acute inflammatory response in either the peritoneal lavage samples or histology sections was also in accord with these data.

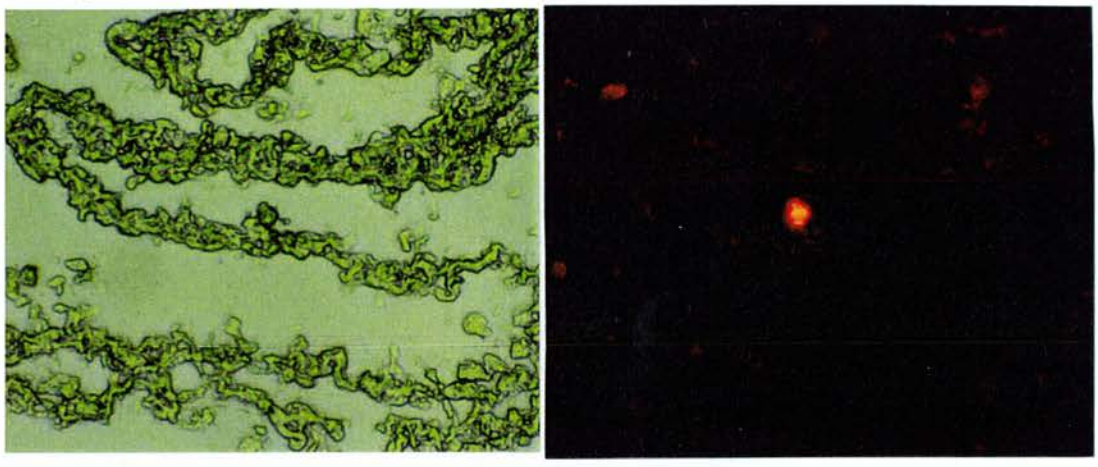
7. 2. 6 Inflammatory Macrophages Emigrate from the Inflamed Site to the Draining Lymph Nodes.

Since the number of live adoptively transferred Mø free in the peritoneal cavity declined rapidly and there was no evidence that they were phagocytosed locally, I sought to identify the tissue fate of these cells. Frozen sections of peritoneum including omentum, a range of lymph nodes, liver, spleen, kidney, lung and heart were examined for PKH26 positive cells. **Table 7. 2** shows the tissue distribution of PKH26-PCL labelled cells with time. There was no evidence of widespread adherence of transferred Mø to the peritoneal lining, with adherent labelled cells confined only to collections on the omentum (**Figure 7. 9**). These collections of varying numbers of PKH26-PCL positive cells, ranging from a few to dozens of cells appear to represent "milky spots" on the omentum (Beelen, 1980). These aggregates did not seem to alter in size or frequency with time being as numerous at 4 hours after transfer as at 1 week.

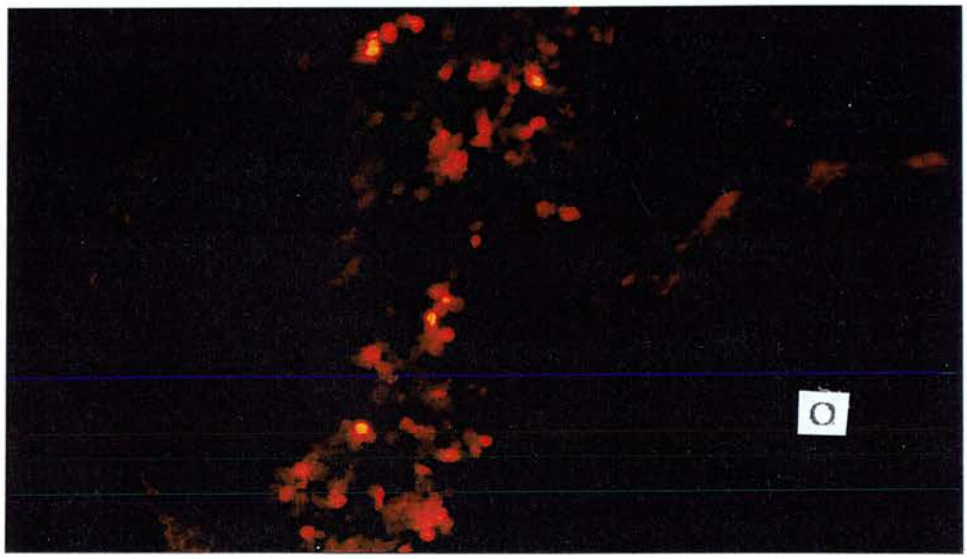
Figure 7. 9 Distribution of PKH26-PCL Labelled Macrophages After Adoptive Cell Transfer.

i) There is No Widespread Adherence to the Peritoneal Membrane.

a) Light Microscopy **b) Red Fluorescent Microscopy**



ii) Macrophage Collection in "Milky Spot" on the Omentum.



Legend for Figure 7. 9 Photomicrographs of frozen tissue sections of recipient mice after adoptive transfer of donor cells (>85% Mø, 36% PKH26-PCL labelled) 72 hours previously. Figure i) a single PKH26-PCL positive cell adherent to a large strip of peritoneum, confirming there is no widespread adherence of donor Mø to peritoneum. Figure ii) shows a large omental collection of labelled Mø in a "milky spot" (MS). Much of the omentum remains free of labelled Mø as outlined in area (O). (x 200).

Table 7. 2

Tissue Distribution of PKH26-PCL Labelled Cells After Adoptive Cell Transfer of *In vivo* Labelled Live Cells.

	Hours After Cell Transfer					
Tissue	**4**	**24**	**48**	**72**	**96**	**1 Week**
Lung	-	-	±	-	-	-
Heart		-	-	-	-	-
Kidney	-	-	-	-	-	-
Spleen	-	sp±	sp±	-	sp±	++
Liver	-	-	sp±	-	-	++
Peritoneum	-	-	-	-	-	-
Omentum	++	++	+++	++	++	++
Lymph Nodes:						
Para-aortic	-	-	±	±	±	-
Mesenteric	-	-	±	±	-	+
Parathymic	-	++	++	+++	+++	+++

Legend for Table 7. 2

The distribution of PKH26-PCL label with time after transfer of 30 x 10^6 donor cells (>85% Mø) into the peritoneal cavity of recipient mice at the same stage of resolving inflammation as the donor mice. Unfixed 3 μM frozen tissue sections from recipient mice were viewed under red fluorescence. "Peritoneum" represented peritoneal lining tissue adjacent to the anterior abdominal wall. A semi-quantitative assessment of PKH26-PCL labelled cell distribution was made thus:

- No PKH26-PCL labelled cells seen/section

± 1 - 3 PKH26-PCL labelled cells seen/section

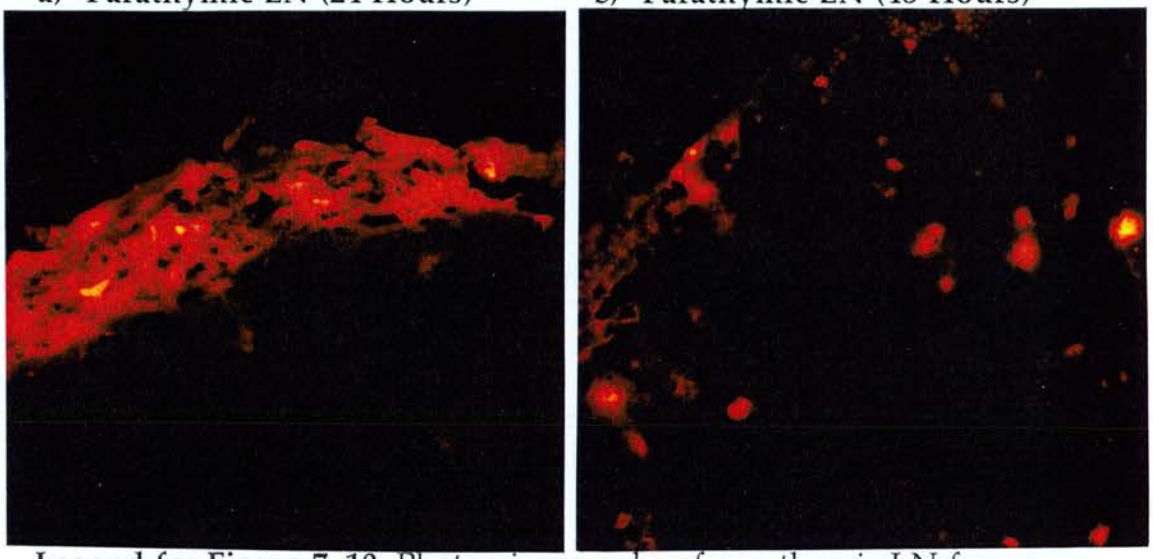
+	4 - 8 PKH26-PCL labelled cells seen/section
++	8 - 15 PKH26-PCL labelled cells seen/section
+++	>15 PKH26-PCL labelled cells seen/section
sp	specks not whole cells

Results are means for 8 mice per time point (4 mice for 4 hour time point). Tissue distribution was the same after transfer of fixed *in vivo* labelled cells (n = 6 mice per time point) and after transfer of *in vitro* labelled cells (n = 6 mice per time point), in neither of these instances was the 4 hour time point performed.

A large number of labelled Mø were detected specifically in the draining LN of the peritoneum, the parathymic LN. The appearance of label did not occur before 4 hours after cell transfer, thereafter labelled cells were detectable for up to 1 week. Despite quantification difficulties there were clearly more labelled cells in the parathymic LN at later time points (72, 96 and 168 hours) than the earlier time points (4, 24, 48 hours) after adoptive cell transfer. These cells were initially located in the subcapsular region, but by 48 hours after cell transfer many cells were also located deep within the node surrounded by lymphocytes (**Figure 7. 10**). The distribution of these cells in relation to B and T cell areas has not been determined yet but they are most likely to be in the medullary cords (Rosen, 1990; Havenith, 1993). There was minimal label detected in other lymph nodes, even those located within the peritoneal cavity, indicating the specificity of the emigration process (**Figure 7. 11**). Label was also detected in the liver and spleen 1 week after cell transfer, at this stage the label was often dim and in small speckles although a few cells were seen (**Figure 7. 11**).

Figure 7. 10 Distribution of PKH26-PCL Labelled Macrophages in the Draining Lymph Nodes.

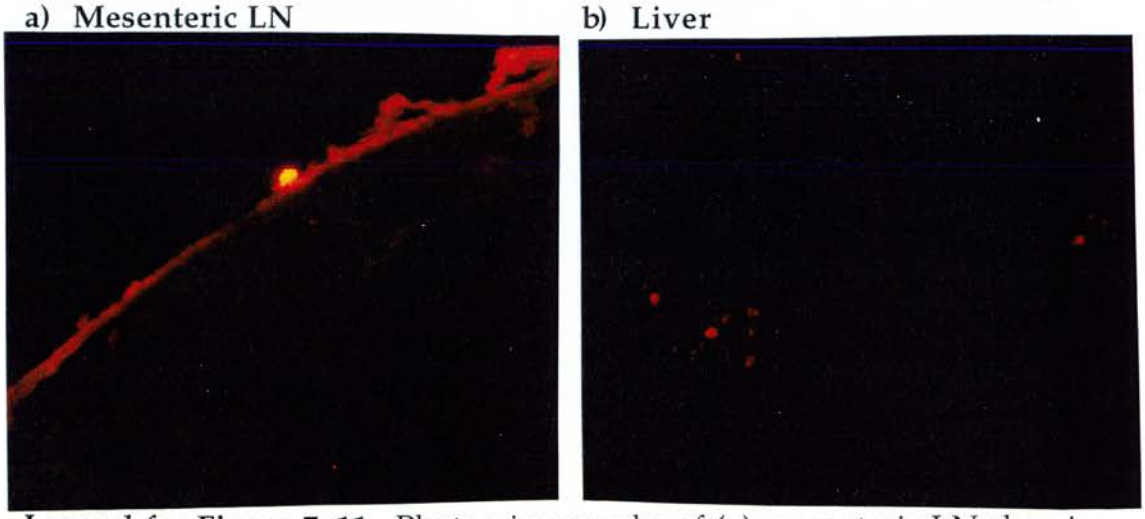
a) Parathymic LN (24 Hours) b) Parathymic LN (48 Hours)



Legend for Figure 7. 10 Photomicrographs of parathymic LN frozen sections from recipient mice after adoptive transfer of donor cells (>85% Mø, 36% PKH26-PCL labelled) **a)** 24 hours and **b)** 48 hours previously. PKH26-PCL labelled Mø are seen initially in subcapsular space of the LN **(a)** but later are widely distributed throughout the node **(b)**.

7. 11 Macrophage Emigration is Specifically to the Draining LN.

a) Mesenteric LN b) Liver



Legend for Figure 7. 11. Photomicrographs of **(a)** mesenteric LN showing a single PKH26-PCL labelled Mø outside the node and none within. **(b)** One week after cell transfer some PKH26-PCL label is seen in the liver, although commonly only specks of red fluorescence as pictured (x 200).

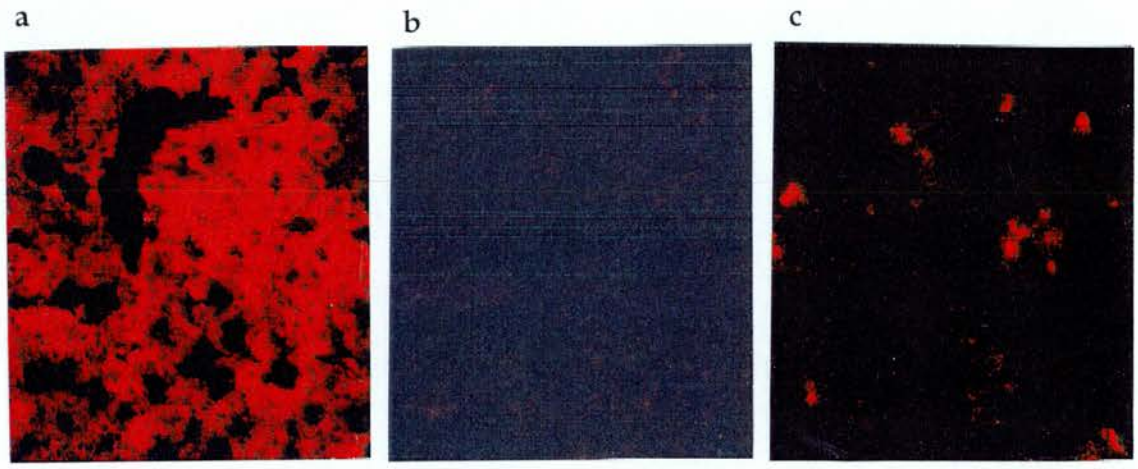
Interestingly, the tissue distribution of PKH26-PCL with transfer of fixed cells was remarkably similar to that observed following transfer of live cells. Again there were collections of PKH26-PCL positive cells on the omentum, commensurate with "milky spots" and the parathymic LN were again specifically and strongly labelled with PKH26-PCL positive cells, late labelling was detected in the liver and spleen as before. No label was seen in the lung, heart, or kidney at any time after transfer of live of fixed donor Mø and only occasional labelled cells were seen in the para-aortic and mesenteric lymph nodes. This indicated that the pattern of emigration of recipient Mø that had phagocytosed donor cells was similar to that of live adoptively transferred donor Mø.

Disrupted spleen and para-aortic lymph nodes, analyzed using flow cytometry to detect PKH26-PCL labelled Mø, showed less than 1% percent of splenic and para-aortic Mø were labelled at any time point. Both mechanical and enzymatic disruption resulted in > 10% trypan blue positive cells, the presence of which made interpretation of dual labelling difficult as the antibodies were non-specifically bound by damaged cells. Parathymic nodes were all used for frozen or paraffin sections hence none were disrupted for flow cytometric analysis.

7. 2. 7 Adoptively Transferred Live Macrophages are Not Phagocytosed Prior to Emigration.

To confirm that adoptively transferred live Mø were indeed emigrating to the parathymic LN and not being carried there passively after being phagocytosed by recipient Mø prior to emigration, frozen sections were examined for the presence of H-2^d.

Figure 7. 12 Donor Cells are Not Phagocytosed Prior to Emigration to the Draining Lymph Nodes.



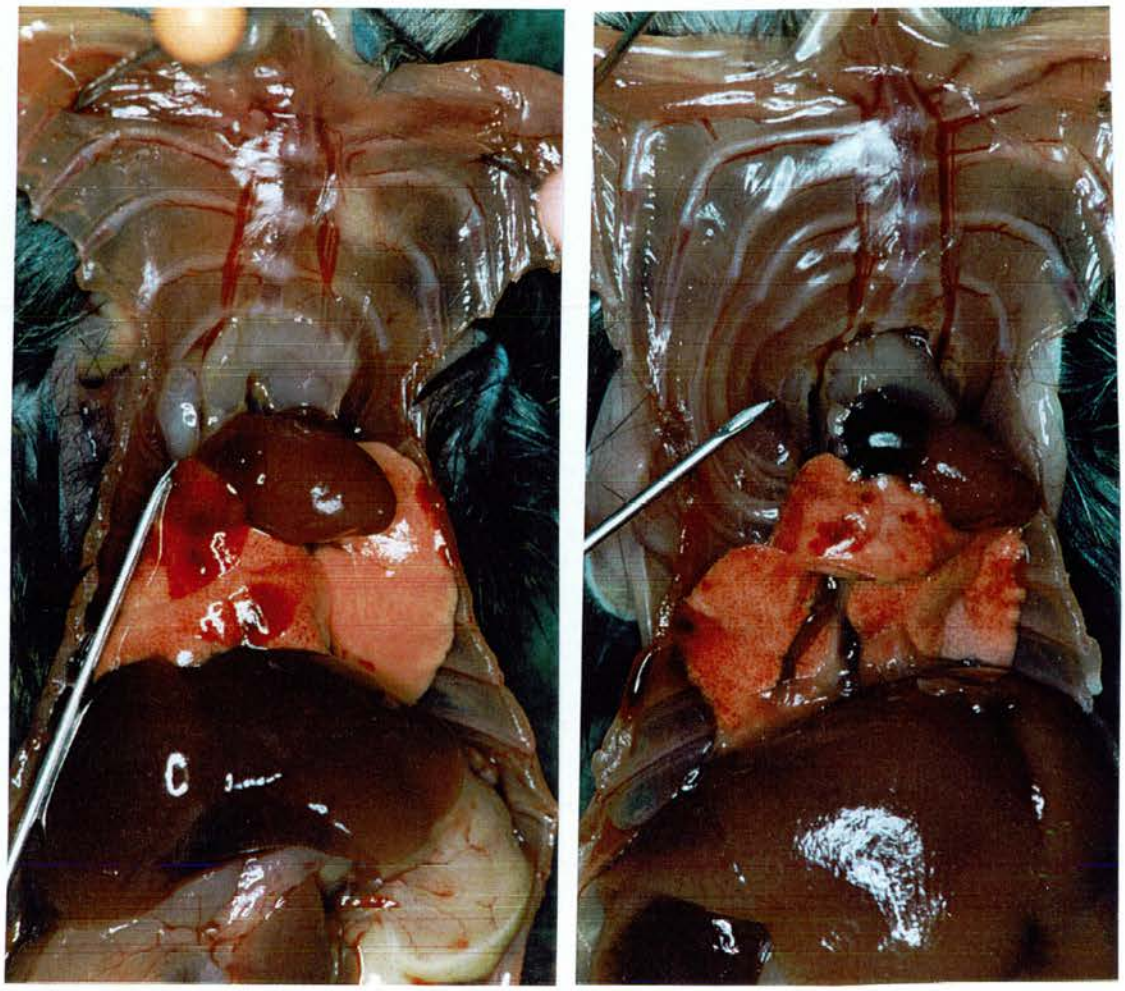
Legend for Figure 7. 12 Photomicrographs demonstrate **(a)** Positive control for H-2^d immunostaining: CB1 parathymic node stained with biotinylated anti-H-2^d, using avidin/biotin-alkaline phosphatase and Vector® red alkaline phosphatase substrate, taken under red fluorescence, demonstrating H-2^d expression on all the cells. **(b)** Parathymic LN from C3HF/KAM mouse as a negative control stained as for **(a)** showing no H-2^d expression. **(c)** H-2^d immunostaining of parathymic node from recipient C3HF/KAM mouse 1 week after adoptive transfer of live H-$2^{k/d}$ donor Mø now showing a number of positive donor cells within the node (all taken at x 200).

As processing for anti-$H2^d$ immunostaining destroyed PKH26-PCL fluorescence, double staining was not possible. Instead serial sections demonstrated that after live cell transfer, H-2^d staining was always and only present when there was PKH26-PCL staining on adjacent sections. The distribution of PKH26-PCL label and H-2^d expression within the nodes was the same. Positive H-2^d staining in the parathymic LN confirmed it was the live donor Mø themselves that had emigrated from the inflamed site (**Figure 7. 12**). In contrast, there was no H-2^d staining in the parathymic LN after return of fixed Mø, despite strong PHK26-PCL fluorescence. These data demonstrate that live donor Mø emigrated intact from the inflamed site to the draining LN whereas fixed Mø were phagocytosed within the peritoneal cavity, a proportion of the phagocytosing Mø then emigrating to the draining LN where they could be detected by the presence of the ingested red fluorescent label. Staining for presence of H-2d in the spleen demonstrated only occasional intact donor cells, these cells were not detected before 48 hours after cell transfer. This suggests that the bulk of the PKH26-PCL fluorescence detected at 1 week did not represent further migration of Mø from the LN.

7. 2. 8 Parathymic Lymph Node Histology.

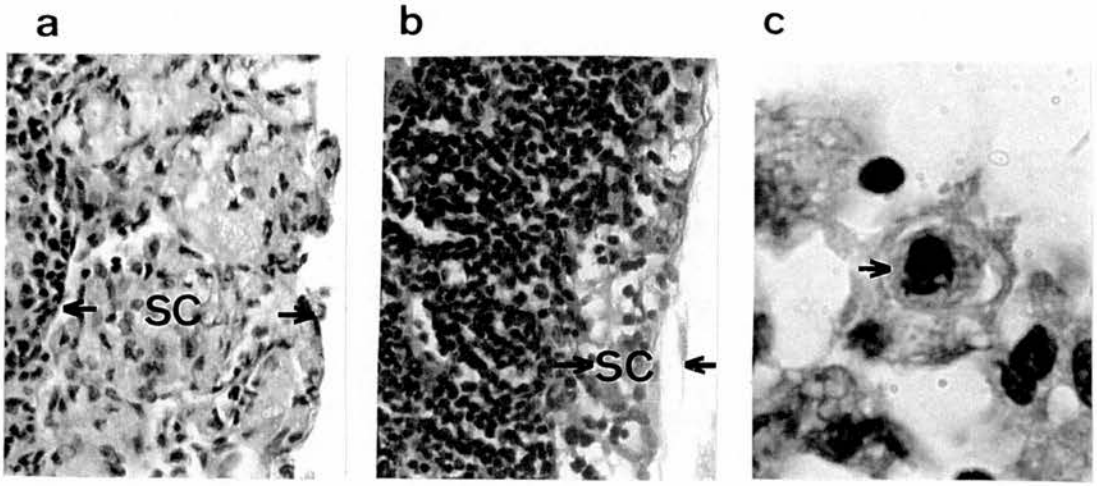
Macroscopically the parathymic lymph nodes were grossly enlarged by 5 days after i.p. TG but regressed steadily to normal size over the ensuing week (**Figure 7. 13**). This was confirmed by the reduction in wet weights of the parathymic lymph nodes from 67 ± 6.8 mg (mean ± SD) at the time of cell transfer to 11.25 ± 10.7 mg 1 week later ($p < 0.01$, $n = 5$).

Figure 7. 13 Macroscopic Changes in Parathymic Lymph Nodes with Acute Inflammation and Resolution of Inflammation.



Legend for Figure 7. 13 Grossly enlarged parathymic lymph nodes (left) five days after i.p injection of TG, compared with normal sized nodes (right) seven days later. Parathymic lymph nodes are indicated with the pointer, they lie lateral to the internal mammary vessels and thymus. In each case the heart is reflected to the left, the lungs deflated and the liver seen below.

Figure 7. 14 Histology of Parathymic Lymph Nodes During Resolution of Acute Inflammation.



Legend for Figure 7. 14 Photomicrographs of H&E stained paraffin sections of (**a**) reactive parathymic LN six days after i.p. injection of TG showing an enlarged subcapsular space (SC) containing many vacuolated Mø (x 100). (**b**) Parathymic LN six days later demonstrating subcapsular space returning to normal with the resolution of inflammation (x 100). (**c**) After transfer of formalin fixed cells, Mø could be seen in the subcapsular space of parathymic LN that has clearly phagocytosed other Mø (arrow) (x 400).

The inflammatory reaction in these LN was consistent with a resolving granulomatous response with several multinucleate giant cells detected per section. Over the first 3 days after cell transfer the subcapsular space was packed with intensely vacuolated Mø (**Figure 7. 14**), these Mø decreased steadily in number and apparent size and the subcapsular space returned to near normality. Throughout the entire time course very low levels of apoptosis could be seen within the LN. There was little evidence of a wave of apoptosis at any particular time and it was not possible to confirm the identity of the apoptotic cells. Transfer of phagocytosed fixed cells from the peritoneal cavity to the parathymic LN was suggested on haematoxylin and eosin sections by the appearance of a number of Mø bearing identifiable Mø within phagocytic vacuoles (**Figure 7. 14**). Histology of lung, liver, kidney, heart and spleen was normal.

7. 2. 9 Comparison of Resident and Inflammatory Macrophage Emigration Kinetics.

A series of preliminary experiments were performed to investigate whether there were different emigration kinetics for resident Mø from the non-inflamed peritoneum and inflammatory Mø from the inflamed site. To establish whether the act of adoptive cell transfer into the non-inflamed peritoneum would in itself induce inflammation, peritoneal lavageates 24 hours after i.p. injection of TG, 5 x 10^6 Mø or PBS alone were compared with no injection at all, determining the number of cells and the percent PMN as indicators of acute inflammation, results are shown in **Table 7. 3**. This shows that the injection of cells into the non-inflamed peritoneum did not cause significant inflammation.

Table 7. 3

Intraperitoneal Injection of Macrophages Causes Minimal Inflammation.

	Total cells (x 10^6) in peritoneal lavage	PMN %	Mice n
No Injection	4.2	1.1	32
i.p TG	13.8	53.0	8
i.p. PBS	6.3	1.2	2
i.p. Mø	7.0	6.8	8

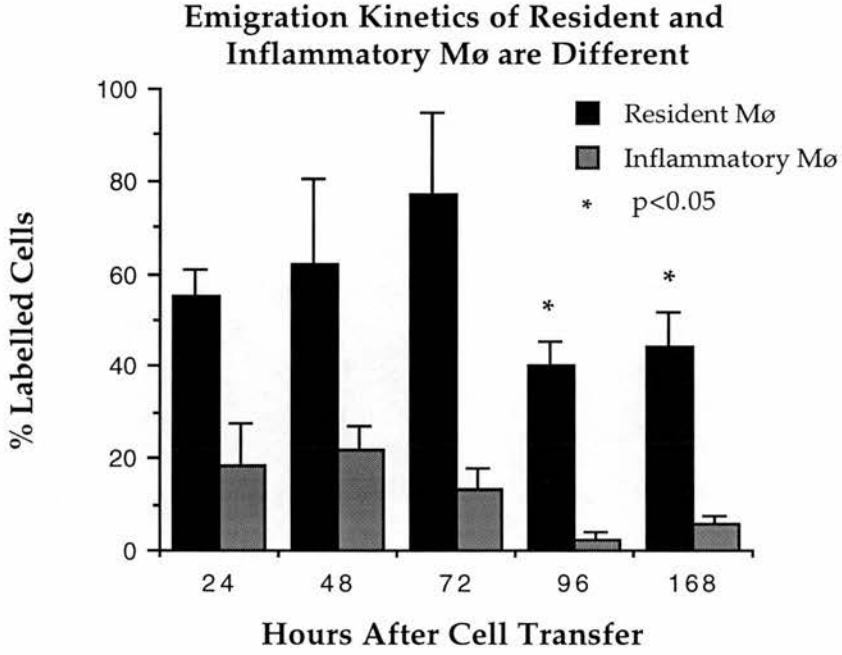
Legend for Table 7. 3

Mice were sacrificed 24 hours after i.p injection and their peritoneal cavities lagaved with 5 mls PBS. Cells were counted by haemocytometer and % PMN determined from cytospin preparations, duplicate slides were prepared for each mouse and 500 cells per slide counted. The % PMN in peritoneal lavage after i.p. Mø injection was variable, 2 mice had 13 and 15% PMN respectively whilst the rest all had < 5%. The total cell number was still very low despite the addition of a further 5 x 10^6 Mø into the peritoneal space.

To determine whether there were differences in the emigration of resident Mø from the non-inflamed peritoneum when compared with inflammatory Mø from the inflamed peritoneum, a comparative study was performed. The peritoneal cavity of mice were lavaged after *in vivo* red fluorescent labelling in the absence of any inflammatory challenge. To increase the Mø labelling, 1 ml of PKH26-PCL dye was used and 3 to 4 x 10^6 cells were recovered in the lavage with 1.5 -2 x 10^6 being labelled Mø. To allow transfer of 4 x 10^6 labelled Mø, 3 donor mice were sacrificed for

each recipient. After semi-allogeneic cell transfer into mice who had also not received any inflammatory challenge, recipients were sacrificed and the percent labelled cells remaining in their peritoneal cavity with time determined, results are shown in **Figure 7. 15** and compared with that of inflammatory Mø transfer into the inflamed peritoneum.

Figure 7. 15



Legend for Figure 7. 15

PKH26-PCL labelled resident, non-inflamed Mø were adoptively transferred into the non-inflamed peritoneal cavity of semi-allogeneic mice. The % labelled cells recovered with time was determined and compared with that of inflammatory Mø after transfer into the peritoneum with resolving inflammation. None of the resident Mø and <10% of the inflammatory Mø were phagocytosed over the course of the experiment. The results were compared using Wilcoxon's rank sum test for non-parametric data, $p<0.05$ taken as significant. Four mice were examined at each time

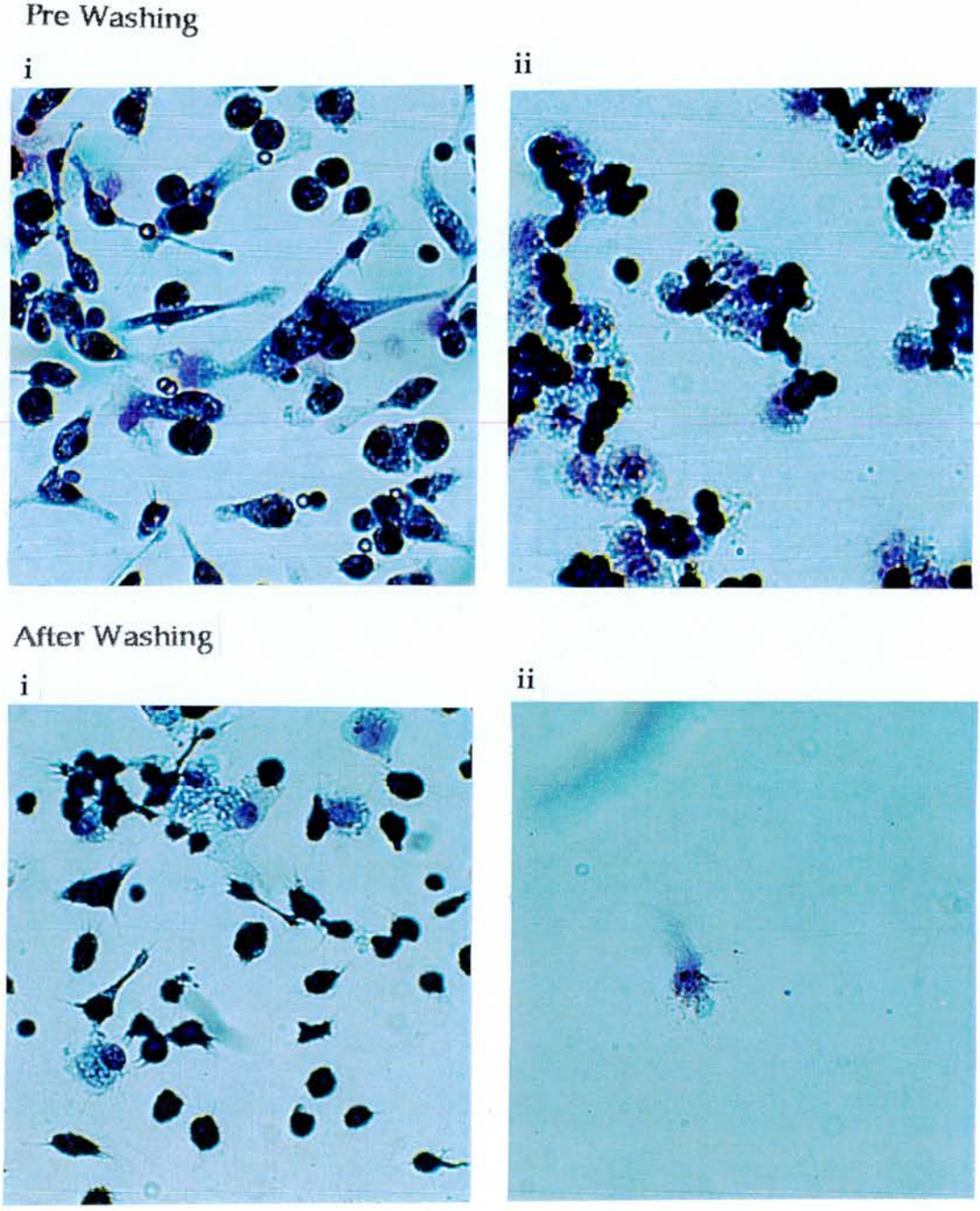
point for the inflammatory challenge and 10 for the resident transfer (except at 48 and 72 hours when 2 mice were examined at each point).

These results suggest that in the absence of inflammation the Mø emigration rate is retarded, consistent with the prolonged turnover time for resident peritoneal Mø reported by others (Rosser, 1970; Haskill, 1985; van Furth, 1989).

7. 2. 10 Effect on Macrophage Emigration of Blocking CD11b.

Mø migration into inflammatory sites has been shown to be dependent on functional expression of specific adhesion molecules including CD11b. Cells leaving the inflammatory site must cross the peritoneal membrane, this emigration may be an active process requiring cell adhesion, as it is for cell recruitment. Stomata between mesothelial cells overlying draining lymphatics dilate with inflammation (Beelen, 1980; Leak, 1983), which could allow direct passage of Mø into lymphatics without necessitating cell adhesion. To begin the process of investigating whether adhesion molecules were important in the emigration of inflammatory Mø from the inflamed peritoneum, I employed the CD11b blocking mAb M1/70 (Pharmingen - low endotoxin, no azide). Titration of this mAb on inflammatory Mø showed that binding was saturated at 0.5 $\mu g/10^6$ cells. The blocking action of this mAb on Mø adhesion was confirmed by preventing adherence to tissue culture plastic (**Figure 7. 16**). In a preliminary experiment inflammatory Mø were elicited with TG and labelled with i.p. PKH26-PCL in standard fashion, 36 x 10^6 of these cells were incubated with 450 µg of M1/70 (a x 25 excess) for 10 minutes at 4°C.

Figure 7. 16 The Antibody M1/70, an Anti CD11b mAb Blocks Macrophage Adherence.



Legend for Figure 7. 16 Suspension cultured Human Mø are adhered to tissue culture plastic **i)** after 30 minutes incubation in medium and **ii)** after 30 minutes incubation in the presence of the mAb M1/70. Cells exposed to medium alone remain adherent after vigorous washing whilst those exposed to M1/70 are non-adherent and all wash off (x 200).

18 x 10^6 cells (>85% Mø <5% trypan blue positive, unwashed hence in the presence of 225 µg antibody/mouse) were then injected i.p. into each of 2 mice who had been injected with TG 5 days earlier, these mice were sacrificed 24 hours later. The dual colour flow cytometry data from peritoneal lavage of the two recipient mice are shown below in **Table 7. 4**.

Table 7. 4

Blocking CD11b on Semi-allogeneically Transferred Donor Inflammatory Macrophages Increases Their Emigration Rate

Quad-stats	**Mouse A**				**Mouse B**			
Region	**Antibody**				**Antibody**			
	D	**cD**	**K**	**cK**	**D**	**cD**	**K**	**cK**
1	2.1	1.4	40.2	0	3.1	0.4	11.2	0.5
2	1.4	1.5	9.6	1.5	5.3	0.1	2.1	0.2
3	86	96.3	50.2	96.2	91.3	98.6	86.7	99.1
4	10.5	0.8	0	2.3	0.3	0.9	0	0.2

		Mouse A	**Mouse B**
PKH26-PCL labelled cells	***(K2 -cK2)***	8.1%	1.9%
H-$2^{k/d}$ cells	***(D1 + D2)- (cD1 + cD2)***	0.6%	7.9%
Phagocytosed donor cells	***(D4 - K4)***	10.5%	0%

Legend for Table 7. 4

Two mice labelled A and B each had 18 x 10^6 cells (>85% Mø) in the presence of 225 µg M1/70 blocking anti CD11b mAb injected i.p. 5 days after i.p. TG. Cells were lavaged from their peritoneal cavities 24

hours later and the % cells labelled with anti-H-2^d and anti-H-2^k mAb (green fluorescence), as well as with PKH26-PCL (red fluorescence) are shown for each mouse. D = Anti H-2^d, cD = Isotype control, K = Anti H-2^k, cK = Isotype control: values shown are percent cells in each region defined by the quad-stats settings. Four regions are identified for each antibody using quad-stats: 1 = green positive, red negative, 2 = green positive, red positive, 3 = green negative, red negative and 4 = green negative, red positive. PKH26-PCL red fluorescent labelled cells were thus {anti-k region 2 - isotype control region 2} whilst H-2^d positive cells were {anti-d region 1 and 2 - isotype control region 1 and 2}. Phagocytosed cells were {anti-d region 4 - anti-k region 4}

These results are inconclusive and require further experimentation, however interesting trends were apparent in the cell kinetics. In mouse A there was significant phagocytosis of adoptively transferred Mø, not encountered with adoptive transfer of live cells in the absence of mAb. Phagocytosis was not observed in mouse B. In both mice the percent of donor cells remaining within the peritoneal cavity after 24 hours (10.5% and 7.9%) is markedly reduced when compared with cells transferred in the absence of M1/70 (31% H-2^d cells recovered at 24 hours). This may suggest that CD11b is important in retaining the Mø at the inflammatory site. Tissue distribution was similar to that reported in **Table 7. 2**, with milky spot collections seen and fluorescent cells detected in the draining lymph nodes of both mice. Anti H-2^d staining confirmed these were donor cells in the parathymic lymph node of mouse B.

7. 3 DISCUSSION.

Although much work has been undertaken on constitutive resident Mø turnover in the non-inflamed "steady state" peritoneum, this is the first detailed study of the fate of the inflammatory Mø during the resolution of inflammation using adoptive transfer of labelled Mø to quantify the cellular kinetics. By contrast with the PMN or mesangial cell, this work provides clear evidence that inflammatory Mø do not meet their fate locally by apoptosis and phagocytosis but that they emigrate from the peritoneum to the draining LN. It has long been known that Mø are able to migrate to lymph nodes (Harmensen, 1985; van Furth, 1992; Lan, 1993; Thepen, 1993) but in this study I demonstrate that this is the major mechanism for the removal of inflammatory Mø from the peritoneum during the resolution of inflammation and that the time course for this clearance is rapid. The emigration process takes longer than 4 hours but most adoptively transferred Mø have been cleared within 96 hours. Upon reaching the LN the cells become localized initially to the subcapsular space, by 48 hours after cell transfer however many cells were detected deeper within the LN, surrounded by lymphocytes. The emigration of the inflammatory Mø occurs specifically to the draining LN, there being little evidence of migration of donor Mø to other LN or other tissues. Although PKH26-PCL label could be detected in the liver and spleen 1 week after transfer this was accompanied by the detection of very few H-2^d positive cells. Hence the draining node apparently represents the "final resting place" for the bulk of the inflammatory Mø. These data suggest that monocyte/Mø undergo a two stage migration process; initially as monocytes they emigrate from the blood to the inflamed site where they fulfill their inflammatory and scavenging roles. Thereafter, a matter of days later in this model of resolving

inflammation, the inflammatory Mø migrate to the draining LN where they would be well positioned to contribute to the generation of an immune response.

Previous studies with tritiated thymidine labelling of dividing cells have given an indication of the local rates of cell turnover in certain inflammatory circumstances (Ryan, 1970; Hopper, 1986). Loss of label from the inflammatory site would not have indicated whether this was due to cell death or emigration. Indeed should there have been significant reutilization of radiolabel subsequent to the phagocytosis of dead labelled cells, cell life-span would have appeared falsely prolonged. Detection of radioactivity at local or distant sites would not necessarily have indicated the fate of the adoptively transferred labelled cells themselves. Moreover, as Mø are capable of significant proliferation, even at the site of inflammation (Coggle, 1982; Bitterman, 1984; Bouwens, 1986), uncertainty about the relative contributions of cell proliferation and cell influx could cause further interpretation difficulties. Our data using a unique dual labelling technique showed that the decline in Mø recovery at the inflamed site was not due to local phagocytosis and that the cells detected distantly in the parathymic LN were indeed live intact donor cells.

The reduction in recovery of donor cells from the peritoneal cavities of recipient mice was not due to a reaction to the presence of foreign antigen ($H\text{-}2^d$), as demonstrated by the similar kinetics for semi-allogeneic and syngeneic cells after adoptive cell transfer. Other workers using allogeneic or semi-allogeneic cell transfer have also found little difference in cell kinetics over the first few days compared with syngeneic transfers

(Bell, 1984). Consistent with this interpretation there was no evidence for a secondary inflammatory response histologically or in the peritoneal lavageates after adoptive cell transfer.

The reduction in recovery of donor cells in the peritoneal cavity with time was also not due to widespread adherence to the peritoneum. Although Mø adhered to the omentum in "milky spot" aggregates, these did not increase in number or size over the time course studied. Hence increased adherence alone was unlikely to account for the observed decrease in donor cell recovery. After adoptive transfer of live Mø there was little evidence of their phagocytosis; the PKH26-PCL labelled cells recovered expressed the donor H-$2^{k/d}$ rather than the recipient H-2^{k} surface antigen. The decrease in the percent donor cells recovered was not due to a dilution effect from the influx of unlabelled cells, since the total number of lavageable Mø also declined, consistent with resolving inflammation.

PKH26-PCL labelled cells were not handled differently from unlabelled cells, the decline in H-$2^{k/d}$ cells being proportional to that of PKH26-PCL labelled cells and both detection methods indicating a steady drop in number of adoptively transferred cells free in the peritoneum. The decline in the percent PKH26-PCL positive cells recovered was not due to loss of the label as demonstrated both by *in vitro* culture and *in vivo* tissue section results. Other groups have confirmed that although labelling intensity declines slowly with time, labelled Mø can still be distinguished from unlabelled Mø for up to seven weeks (Melnicoff, 1988b and 1989). In conjunction with data demonstrating the accumulation of labelled cells within the draining LN, this suggests that

emigration of the inflammatory Mø from the peritoneum was the main mechanism responsible for the clearance of these cells.

In contrast to the results for live cell transfer, transferred formalin-fixed cells were phagocytosed quickly and efficiently, with very few remaining free by 24 hours, suggesting efficient clearance of dead or effete cells by inflammatory Mø. This observation also confirmed that TG elicited Mø were effective phagocytes, despite reported differences in their bactericidal capacity when compared with Mø induced by other inflammatory stimuli, (Hopper, 1986). The kinetics of removal of the phagocytosed fixed transferred cells was slower than that of the live transferred cells. Recipient Mø remained functionally intact after phagocytosing formalin-fixed donor cells, since they were able to emigrate to the draining LN. Thus it was unlikely that the delay in clearance of labelled cells was due to toxicity of the formalin-fixed donor cells. A sub-population of phagocytic Mø with slower emigration capability was also unlikely since the populations of donor and recipient cells were elicited in the same way. An alternative and plausible explanation is that phagocytosis of the killed transferred Mø activated the phagocytosing Mø in a manner which prolonged their presence at the inflamed site. We have previously shown *in vitro* that Mø uptake of opsonized cells (Meagher, 1992) and necrotic eosinophils (Haslett, 1994) caused Mø activation and secretion of thromboxane B_2 and other pro-inflammatory mediators which did not occur when Mø ingested apoptotic cells (Meagher, 1994).

Experiments using starch as an alternative inflammatory stimulus showed that the time course of Mø accumulation and the clearance pattern appeared identical to that of TG elicited Mø. These findings are

likely to be broadly relevant to the mechanisms by which acute inflammation resolves.

The rate of turnover for resident peritoneal Mø in the "steady state" reported in the literature differs markedly from the data presented here for inflammatory Mø during the resolution of sterile inflammation. This was corroborated by the experiments showing that resident Mø transferred into the non-inflamed peritoneum had significantly slower emigration kinetics than inflammatory Mø from the inflamed site. Previous work on the kinetics of resident peritoneal Mø suggested a mean turn-over time of 14.9 days (van Furth, 1989). Adoptive transfer of radiolabelled resident peritoneal Mø indicated that even 2 weeks after cell transfer more than half the labelled cells could be recovered in peritoneal lavage (Rosser, 1970). More recent experiments using fluorescent labelled resident peritoneal Mø are somewhat contradictory, one group using an *in vitro* labelling technique reported rapid decline in cell retrieval (Rosen, 1990) whilst others using *in vivo* labelling again demonstrating a prolonged half life of several weeks or more for the resident peritoneal Mø population (Haskill, 1985; Melnicoff, 1988b). The reasons for the differences between emigration kinetics of resident and inflammatory Mø are unknown. Differences may be related to cell factors such as adhesion molecule expression, altered sensitivity to chemotactic agents or to changes in cell mobility, an assertion which might be tested further using adoptive transfer of inflammatory Mø into the non-inflamed peritoneum. Differences in emigration kinetics may represent a potential control point in the resolution of inflammation. To study this further transfer of green fluorescent resident Mø and red fluorescent inflammatory Mø into the non-inflamed and inflamed peritoneum

would allow determination of the degree to which cell and environmental factors control emigration kinetics. Preliminary data on blocking CD11b function is exciting as it raises the possibility that this molecule may be important in retarding Mø emigration. It is worth noting that the anti-CR3 blocking mAb 5C6 caused apoptosis in microglia (Reid, 1993). Whether the anti-CR3 mAb M1/70 is capable of inducing apoptosis is unknown, this needs to be borne in mind in view of the rapid phagocytosis of live donor cells in mouse A (section 7. 2. 10).

The tissue distribution of PKH26-PCL labelled cells after leaving the inflammatory site is in accordance with that reported previously for resident Mø emigration (Rosser, 1970; Rosen, 1990). The lymphatics draining the peritoneum arise from the sub-diaphragmatic surface, entering ducts running with the internal mammary vessels to reach the anterior mediastinal LN. Efferent lymphatics from these usually run to the right lymphatic duct, with some cross-communication to the thoracic duct (Yoffey, 1970). Elegant electron microscopy studies have confirmed that stomata exist between the mesothelial cells overlying lymphatic spaces and these widen during inflammation allowing for cell emigration (Beelen, 1980; Leak, 1983). Within the parathymic LN themselves, the PKH26-PCL labelled cells were initially distributed in the subcapsular region, with many labelled cells present deep in the node 48 hours or more after cell transfer. This is in accordance with previous work on resident peritoneal Mø emigration; cells being detectable in the outer cortex and medullary cords of LN (Rosen, 1990; Soesatyo, 1993). Mø migration to LN has been demonstrated in other systems (Harmensen, 1985; Lan, 1993; Thepen, 1993, van Vugt, 1993b) but quantification of rates of emigration and cell death has not been performed. The distribution

pattern of inflammatory Mø in the draining LN would allow for antigen presentation to lymphocytes. Since Mø are widely recognized as "professional" antigen presenting cells, their emigration to the LN where interaction with immunocompetent cells is maximized, may well have functional implications beyond that of simple cell clearance. This need not necessarily be to augment immune responses. The presence of Mø along with dendritic cells has been shown to down-modulate lymphoproliferative responses *in vitro* (van Vugt, 1993a).

It is interesting to review this work in the light of what is known of the fate of the PMN with resolving inflammation. The PMN is a short lived cell that dies at the site of inflammation where it is phagocytosed by the inflammatory Mø (Haslett, 1994). Similarly the mesangial cell, a myelofibroblast-like cell with phagocytic potential in the kidney, has been shown in experimental inflammation to die in significant numbers by apoptosis and to be cleared by local phagocytosis (Baker, 1994). In contrast the inflammatory Mø does not seem to die locally at the inflamed site but rather, after migration into the inflamed site, emigrates non-randomly to the draining LN, a situation more analogous with that of lymphocyte trafficking or Langerhans and dendritic cell migration (Austyn 1990; van Vugt, 1993b). The clearance of inflammatory Mø from the site of acute inflammation is rapid, large numbers of Mø emigrating daily during resolution. This two stage migration process in the life-span of the inflammatory Mø is well suited to phagocytosis of materials at the inflamed site and subsequent antigen presentation in the LN and thus may have implications for the initiation and amplification of immune responses against agents derived from the inflamed site.

Chapter 8

CONCLUSIONS AND SPECULATIONS.

CONCLUSIONS AND SPECULATIONS.

In this thesis I have examined a number of aspects of the cell biology of mononuclear phagocytes relevant to the process of the resolution of inflammation. In particular I have examined the fate of the inflammatory Mø both *in vitro* and *in vivo*, neither of which had hitherto been studied in detail. At the outset of this project only Mangan *et al.* had documented monocyte apoptosis (Mangan 1991 a and b) and there were only two reports on Mø apoptosis (Waring, 1990; Hogquist, 1991a). Robust methods for identifying and quantifying apoptotic monocytes and Mø in both suspension and adherent culture were not established and no comparison of the sensitivity to apoptosis of these cells had been made. Furthermore the physiological relevance of Mø migration from tissues and inflamed sites to LN and to other tissues such as the gut and its significance as a clearance mechanism for inflammatory Mø were unknown. Although several groups had studied monocyte migration into inflamed sites (Issekutz, 1981; Rosen, 1990, van Furth, 1992) there was no data on the fate of the inflammatory Mø. My aim was to study the regulation of cell death in monocytes and Mø which may be a critical factor determining the extent of Mø accumulation in tissues, to characterize the process of apoptotic cell death in mononuclear phagocytes in terms of cell surface phenotype and physical parameters and to look at potential mechanisms controlling Mø longevity. I also wished to gain some insights into the *in vivo* mechanisms of clearance of inflammatory Mø from tissues.

Other phagocytic cells (PMN and mesangial cells) have been shown to die by apoptosis and become ingested by Mø during the resolution of

inflammation (Savill, 1989; Baker, 1994). Mø, in contrast, are believed to be long lived cells, a suggestion supported by data on the kinetics of resident tissue Mø and on studies of chronic inflammatory lesions (van Furth, 1992). Mø are now known to undergo apoptosis in response to a number of different stimuli, including agents known to be present at the inflamed site such as pathogenic bacteria (Zychlinsky, 1994), NO· (Albina, 1993) or silica (Sarih, 1993b), however the extent to which Mø apoptosis contributes to Mø clearance had not been addressed. Monocytes are sensitive to pro-apoptotic stimuli (Mangan, 1991a and b) and I hypothesized that there may be differences in the survival characteristics between these two related cells although no direct comparison had been made. In this work, I have shown that monocytes are more sensitive to the induction of apoptosis than Mø and that certain physiological agents (serum and TNF-α) can prolong monocyte survival (Chapter 4). Mø are much more resistant to the induction of apoptosis, with significant differences in the constitutive losses between monocytes and Mø in culture as well as in the pro-apoptotic effects of serum withdrawal (Chapter 4) and ROI (Chapter 6) on these cells.

With the onset of apoptosis there were specific changes in surface molecule expression of monocytes, Mø and U937 cells (Chapters 3 and 5). A range of cytokine receptors and CD14 were expressed at reduced levels whilst certain receptors were retained late into the apoptotic process including Fas and IFNγR, this pattern was independent of the method by which apoptosis was induced. Apoptosis in monocytes and Mø could be induced by protein synthesis inhibition (Chapter 3), suggesting that there may be continued synthesis of a short-lived anti-apoptotic protein by these cells. Interestingly, although the oncogene product Bcl-2 has been

shown to protect a wide range of cells from the induction of apoptosis (Jacobson, 1995), only low levels of Bcl-2 were detected in monocytes and these fell even further with maturation into Mø (Chapter 6). Expression of *c-myc* and *abl* also failed to correlate with monocyte and Mø apoptosis. The comparative resistance of Mø to ROI induced apoptosis was shown, these cells requiring 60 fold more H_2O_2 than HL60 cells to induce the same degree of apoptosis, a difference which may be explained by the endogenous anti-oxidant capacity of Mø. Whether a difference in anti-oxidant capacity accounts for the differences between monocyte and Mø sensitivity to apoptosis remains to be determined but the sensitivity of monocytes to ROI induced apoptosis was greatly increased in the absence of serum which provides some anti-oxidant capacity.

During the course of this work a number of papers have been published on agents that induce Mø apoptosis (Chapter 3). Recently studies have also focused on mechanisms controlling Mø survival, with descriptions of specific activation induced apoptotic pathways (Munn, 1995; Bingisser, 1996) and of monocyte expression of the oncogene product Bcl-2 (Iwai, 1994) and of Fas (Richardson, 1994). These studies corroborate many of the findings of this work, demonstrating that sensitivity to apoptosis is down-regulated during monocyte differentiation into Mø. With hindsight it would have been interesting to include IL 10, IFN-γ and MIP-1α in studies on monocyte and Mø survival and the induction of apoptosis. Although survival characteristics of these cells were studied in suspension and in adherent cultures, further time would have allowed the effects of exposure to different matrix components to be determined and the effect of prior cell activation on the induction of apoptosis to be investigated.

Having characterized apoptosis in both monocytes and Mø and demonstrated that Mø are less susceptible to the induction of cell death, I then investigated the physiological significance of this in the clearance of inflammatory Mø from the inflamed site. This work establishes that unlike PMN and mesangial cells, Mø are not cleared by local phagocytosis (Chapter 7). Rather, having performed their pleiotropic roles during inflammation, Mø emigrate specifically to the draining LN during the resolution of inflammation. There was minimal local phagocytosis of Mø at the inflamed site and also little further emigration from the lymph nodes to other more distant sites within the time course studied. Thus, although the Mø is clearly capable of undergoing apoptosis, it seems that Mø clearance from the inflamed site is by active emigration rather than cell death. The kinetics of Mø clearance from the inflamed and non-inflamed site are very different, with inflammatory Mø exhibiting significantly faster rates of emigration than resident Mø (Chapter 7). The mechanisms by which these differences occur are unknown and are the subject of continued investigation.

Putting this work into context with other developments in the field it is possible to view the circulating monocyte as a relatively short lived cell which dies by apoptosis in the absence of pro-survival stimuli (Mangan, 1991a and b; Munn, 1995). Tissue Mø are relatively long lived cells that may either die locally or emigrate to the draining LN (van Furth, 1992; Bellingan, 1996a and b). For peritoneal Mø, emigration has been shown to be the major route of clearance, the situation for other resident Mø remains to be clarified. Tissue Mø can be replaced by self replication or by influx of circulating monocytes (Volkman, 1976; Bouwens, 1986; van Furth, 1992; Ginsel, 1993). It may be that in the absence of inflammation

the usual fate of monocytes is to die and that the bulk of tissue Mø replacement is by self replication, the relative extent to which monocytes replace tissue Mø losses is still a matter for debate (Ginsel, 1993). During acute inflammation monocytes are rapidly called into the inflamed site. Inflammatory cytokines act to promote monocyte survival and their differentiation into Mø (Dougherty, 1984; Mangan, 1991a and b; Leenen, 1993; Munn, 1995). Having orchestrated successful clearance of the inflammatory agent, Mø promote resolution by phagocytosing senescent PMN (Savill, 1989a; Haslett, 1994; Ren, 1994) and emigrating to the draining LN (Bellingan, 1996a). The controls on Mø emigration and the reasons for Mø persistence in chronic inflammation await elucidation. Exciting glimpses at possible controlling factors are seen with the data on blocking CD11b function leading to enhanced Mø emigration and on the differences between resident and inflammatory Mø emigration kinetics (Chapter 7). Whether certain stimuli such as pathogenic bacteria (Zychlinsky, 1991, 1992, 1994), or overwhelming ROI (including NO·) concentrations (Albina, 1993) can alter clearance mechanisms by inducing local Mø apoptosis remains to be seen. It would be attractive to speculate that acute inflammation resolves appropriately without significant disruption of tissue integrity when Mø are capable of clearing the inciting stimulus without any significant toxicity to themselves. However, an excessive or particularly pathogenic inflammatory stimulus may overwhelm Mø protective mechanisms and alter susceptibility to apoptosis resulting in Mø death locally, a failure in clearance and as a consequence, persistent inflammation as occurs with the induction of systemic inflammatory responses leading to the acute respiratory distress syndrome or septic shock (Bone, 1992).

Mø which have emigrated to the draining LN during resolving inflammation are well positioned to interact with other immune cells. The fate of Mø thereafter remains to be determined but during the first twelve days following inflammatory stimulus there was little evidence that Mø accrued in other sites in significant numbers. It may be that the LN does represent their final resting place although this has not been established. The unique architecture of the lymph node promotes interaction of antigen presenting cells and T cells (Gretz, 1996). It would be an attractive notion that, having interacted with cells in the LN, Mø receive a signal to die by apoptosis and in this regard Mø expression of Fas is of interest. In common with other investigators, I demonstrated that anti-Fas mAb were not capable of inducing Mø apoptosis, however these cells may require activation before ligation of Fas can induce apoptosis (Schattner, 1995; Rovere, 1996). Thus it may be possible that inflammatory Mø recently arrived at the draining LN may be primed to receive a pro-apoptotic signal in this way. Both activated T cells and cytotoxic T cells express the Fas ligand and interaction of Fas ligand with Fas can lead to apoptotic cell death (Lowin, 1994, Liles, 1996). This may be a route for elimination of Mø after appropriate interaction with immune competent cells; indeed cytotoxic T cell induction of Mø apoptosis has already been demonstrated (Hogquist, 1991a).

This thesis raises a number of interesting questions that could be addressed in future experiments. The mechanism of the increased resistance of Mø to the induction of apoptosis by oxidants and the possible role of antioxidants in this can be addressed by determining the relative contribution of endogenous antioxidants to protection of the cells against oxidant stress. Potential protective antioxidant mechanisms such as

glutathione and enzymatic detoxification pathways could be blocked by employing specific inhibitors and comparison then made between monocyte and Mø survival characteristics. Patients with chronic granulomatous disease are deficient in superoxide production and the use of monocytes and Mø from these subjects would also help to elucidate the role of these free radicals in inflammatory cell death.

With the wider range of probes for members of the bcl-2 family and other proto-oncogenes now available, the genetic basis of this increased Mø resistance to apoptosis could be further investigated. The potential requirement for Mø activation prior to Fas induced apoptosis can be investigated and the possibility that this is the mechanism of activated T cells promoting Mø apoptosis can be determined in co-culture experiments.

Of considerable interest is the differential emigration kinetics of resident and inflammatory Mø (Bellingan, 1996b). The mechanisms underlying this are open to investigation in a number of ways. Resident and inflammatory Mø can be differentially labelled, mixed and introduced into the non-inflamed or inflamed peritoneum to identify whether cell or environmental characteristics are responsible for the different rates of emigration observed. The *in vivo* use of blocking antibodies to a wide range of adhesion molecules, as described in Chapter 7 for CD11b, is an obvious route to follow. The effect of these mAb on Mø emigration can be determined and the expression of specific adhesion molecules on mesothelium and "milky spots" in non-inflamed and inflamed conditions demonstrated. A different approach would be to use gene knockout mice (specific knockouts of CR3 and L selectin already exist) and

use of these for Mø migration studies would be potentially less problematic than employing blocking antibodies. The state of activation of adoptively transferred Mø can be altered by pre-exposure to potentially activating (TNF-α, IL-1, IL-6) or "deactivating" cytokines such as IL-10 (Bogdan, 1991) and IL-4 (Gautam, 1992; Standiford, 1993) or by incubating Mø in supernatant from acute or resolving inflammatory lesions. Likewise the effect of phagocytosis on Mø emigration can be investigated, we postulate that phagocytosis of apoptotic cells will not materially alter Mø emigration kinetics whilst phagocytosis by Fc or complement mediated mechanisms will activate Mø and delay emigration. As demonstrated in Chapter 7, phagocytosis of fixed cells delayed Mø emigration and it has been previously shown that Mø uptake of apoptotic PMN does not stimulate the phagocytosing Mø (Meagher, 1992). It would have been interesting to using PKH labelled rather than fluorescent microsphere labelled Mø (section 3. 2. 10) to determine the rate and mechanism of Mø phagocytosis of apoptotic Mø. It has been reported that anticoagulants, in particular heparin can alter the Mø disappearance reaction (MDR) (Nelson, 1965; Barth, 1995). The MDR is a physiological response of Mø - both resident and inflammatory - whereby Mø "disappear" from free peritoneal fluid in response to a number of stimuli. It has been postulated to involve Mø adherence and formation of fibrin aggregates, heparin prevents this "disappearance" reaction. The effect of heparin could be investigated on resident Mø and on Mø in acute and chronic inflammation to determine if it has any effect on emigration kinetics. Finally thoracic duct cannulation (Bell, 1984) would facilitate collection of emigrating Mø, enabling accurate quantification of cell trafficking and collection of Mø for analysis of their functional capacity and surface phenotype. It would be of interest to determine the effect of

these emigrating Mø on lymphocyte responses, focusing on antigen presentation and on effects of lymphoid cells on Mø survival. A comparison of Mø emigration responses to known stimuli of acute versus chronic inflammation may help elucidate the reasons for Mø persistence at the site of unresolving inflammation.

Summary of Findings.

In this work I have demonstrated that the Mø is a robust cell which, during its maturation from the monocyte, has gained considerable resistance to the induction of apoptosis. Although these cells can be induced to undergo apoptosis and this process can be characterized in terms of specific surface molecular changes, apoptosis does not represent the normal physiological mechanism of clearance of Mø from the inflamed site. Rather Mø can be shown to emigrate specifically to the draining lymph nodes, inflammatory Mø emigrate rapidly whilst resident Mø follow the same pathways at a much slower rate. I have considered the implications of these findings and further experiments to elucidate the potential underlying mechanisms controlling Mø survival and the controls on their clearance.

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Appendix 4: ENCLOSED PAPERS.

1) Hannah, S., Mecklenburgh, K., Rahman, I., Bellingan, G.J., Greening, A., Haslett, C. and Chilvers, E. (1995) *FEBS Letts.* **372,** 233-237. Hypoxia prolongs neutrophil survival in vitro.

2) Bellingan, G.J., Caldwell, H., Howie, S.E.M., Dransfield, I. and Haslett, C. (1996) *J. Immunol.* **157,** 2577-2585. In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally but emigrate to the draining lymph nodes.

In Vivo Fate of the Inflammatory Macrophage During the Resolution of Inflammation

Inflammatory Macrophages Do Not Die Locally, but Emigrate to the Draining Lymph Nodes[1]

Geoffrey J. Bellingan,[2,3]* Helen Caldwell,* Sarah E. M. Howie,† Ian Dransfield,* and Christopher Haslett*

The resolution of acute inflammation requires bulk clearance of extravasated inflammatory cells in an ordered manner. Neutrophils undergo apoptosis and are ingested by macrophages (Mϕ) via a novel recognition mechanism that fails to provoke proinflammatory responses. Thereafter, the fate of inflammatory Mϕ themselves remains unclear. We investigated this in vivo, developing a semiallogeneic adoptive transfer system to track the fate of inflammatory Mϕ in a murine model of resolving peritonitis. Fluorescently labeled Mϕ from H-2$^{k/d}$ mice were transferred into the peritoneal cavity of H-2^{k} mice at the same stage of resolving inflammation as the donor mice. Dual color flow cytometry permitted discrimination among donor cells, recipient cells, and donor cells that had been phagocytosed by recipient Mϕ. Despite the absence of significant local phagocytosis, the number of transferred Mϕ free in the peritoneum of recipient mice declined rapidly, being undetectable by 96 h. These data suggest that inflammatory Mϕ normally emigrate rapidly from the peritoneal cavity during the resolution of inflammation, contrasting with resident Mϕ, which persist in the noninflamed peritoneum for weeks. Accordingly, labeled nonphagocytosed cells were detected in the draining lymph nodes, but not in a variety of other tissues. Thus, unlike the polymorphonuclear leukocyte, which dies by apoptosis and is ingested by Mϕ, the inflammatory Mϕ itself does not die locally. Having performed its acute inflammatory and scavenging roles, it emigrates in a nonrandom fashion to the draining lymph node, where it may play an important part in the presentation of Ags from the inflamed site. *The Journal of Immunology,* **1996, 157: 2577–2585.**

The inflammatory macrophage (Mϕ)[4] plays a central role in the inflammatory process, being able to secrete and respond to a range of powerful cytokines that control key events in the initiation, resolution, and repair processes of inflammation, but its fate during the resolution of inflammation is unknown (1). The resolution of acute inflammation is characterized by the clearance of extravasated neutrophils (PMN) and Mϕ and the return of normal tissue architecture. However, in a number of disease processes inflammation does not resolve; rather, it persists and is often associated with fibrosis and loss of organ function, as exemplified by chronic bronchitis, emphysema, glomerulonephritis, or rheumatoid arthritis (1, 2). The mechanisms underlying the development of chronic inflammation are poorly understood, but much attention has been focused on the role of the inflammatory Mϕ. Although Mϕ are able to debride tissue and promote wound healing (3), continued Mϕ accumulation is also a hallmark of chronic inflammation. The Mϕ can damage tissue by the release of histotoxic enzymes, proinflammatory and profibrogenic cytokines, or the presentation of Ag to T cells (1, 4, 5). Thus, definition of the mechanisms controlling not only the influx but also the persistence and removal of inflammatory Mϕ are important to our understanding of the pathogenesis of both acute and chronic inflammation.

The origin and influx of monocytes have been well documented (6–8), while the fate of the inflammatory Mϕ has received comparatively little attention. Inflammatory Mϕ are known to be derived from circulating monocytes that migrate into acute inflammatory reactions, mixing with the resident Mϕ population (9). The kinetics of resident tissue Mϕ in the noninflamed steady state have also been the subject of investigation (7, 10), but resident and inflammatory Mϕ differ in cell physiology and activation status (11–14) and may differ in ontogeny (15–17); hence, the direct relevance of studies on the resident Mϕ to our understanding of the tissue kinetics of the inflammatory Mϕ is uncertain.

Some insights into the cellular mechanisms underlying resolution of inflammation have been provided recently by studies relating to the fate of extravasated PMN during the resolution of inflammation. These cells have been shown to undergo apoptosis (18, 19) and become ingested by inflammatory Mϕ using a novel recognition mechanism (20, 21) that fails to stimulate the release of proinflammatory mediators (22). Thus, it has been suggested that this process may represent a nonphlogistic mechanism for the silent clearance of large numbers of PMNs and their potentially histotoxic contents during the resolution of acute inflammation (23). Mϕ in vitro have also been shown to undergo apoptosis in

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[4] Abbreviations used in this paper: Mϕ, macrophage; PMN, polymorphonuclear leukocyte; LN, lymph node; TG, thioglycolate; NMS, normal mouse serum.

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response to a number of stimuli (24–29). Hence, it was entirely conceivable that, in a fashion analogous to the PMN or another phagocyte, the mesangial cell (2), the inflammatory Mϕ, might undergo apoptosis in large numbers locally and themselves become ingested by local phagocytes. However, exhaustive histologic examinations of resolving inflamed sites in the lung and synovial cavity (C. Haslett, unpublished observations) provided no visual evidence of the occurrence of such a process and suggested that the inflammatory Mϕ might meet its fate at a distant site.

To address this question we examined the kinetics of the inflammatory Mϕ using two independent labeling systems. Inflammatory Mϕ from H-$2^{k/d}$ donor mice were labeled with a red fluorescent dye and transferred into the peritoneal cavity of recipient H-2^k mice at the same stage of resolving inflammation as the donor mice. The use of fluorescent tracking dyes is now a well established technique allowing the stable labeling of cells in a nontoxic manner without apparently altering normal cell physiology (30, 31). Immunofluorescence analysis using a FITC anti-H-2^d Ab provided the second method of distinguishing donor or recipient cells, and dual color flow cytometry allowed identification of donor Mϕ that had been phagocytosed by recipient cells.

In the noninflamed peritoneal cavity, the half-life of resident Mϕ has been estimated to be 2 wk or longer (13, 14). In our studies we show that during the resolution process, adoptively transferred inflammatory Mϕ rapidly emigrate intact from the inflamed peritoneum specifically to the draining LN, and this emigration is complete by 96 h, with little evidence of death or engulfment of Mϕ at the inflamed site.

Materials and Methods

Mice and inflammatory challenge

BALB/c (H-2^d), C3HF/KAM (H-2^k), and F1 hybrid CB1 mice (H-$2^{k/d}$) breeding colonies were established locally. Female CB1 mice were used as donor mice, and female C3HF/KAM were used as recipients; all mice were used at 11 to 14 wk of age. The kinetics of the inflammatory cell response to the i.p. injection of 2 ml of thioglycolate (TG; Difco, East Molesey, U.K.) or 2 ml of 1% starch (Sigma Chemical Co., Poole, U.K.) was followed for 21 days (days 3, 5, and 10 for starch) to identify the resolution phase of inflammation in both CB1 and C3H/KAM mice. A minimum of four mice per strain were terminally anesthetized with ether at each time point, and total cell number, viability, and differential counts were determined in the peritoneal lavagates.

Adoptive cell transfer

Peritoneal Mϕ were labeled in vivo with the red fluorescent dye PKH26-PCL (Sigma Chemical Co.). A freshly prepared sterile solution of dye (0.5 ml of 0.5 μM) was injected i.p. into donor mice 1.5 h before peritoneal lavage. Control mice received PBS injection. For adoptive cell transfer experiments, peritoneal inflammation was induced by i.p. TG (or starch) in both donor and recipient mice 5 days before cell transfer. Inflammatory cells (>85% Mϕ; >95% viable), 1.5 h after PKH26-PCL labeling, were lavaged from the peritoneal cavity of the donor mice using 5 ml of sterile PBS (Life Technologies, Paisley, U.K.). Cells were washed twice (250 g) and counted, and viability was determined by the ability to exclude trypan blue. For certain experiments, washed, formalin-fixed cells were employed. Transferred cells (live or fixed) in 2 ml of PBS at the concentrations stated were reinjected into the peritoneal cavity of recipient mice (at the same stage of inflammation as the donors). Recipient mice were killed at various times between 4 h and 1 wk later, their peritoneal cavities were lavaged, and tissue was taken for histology and fluorescence studies. Some mice received unlabeled inflammatory Mϕ to serve as controls for flow cytometry and tissue studies.

Tissue and cell staining

Tissue for frozen sections was immediately snap-frozen in liquid nitrogen, 3-μm sections were cut and mounted unfixed on microscope slides, and the distribution of the PKH26-PCL label was determined using an Olympus BH-2 RFCA fluorescent microscope. Staining for the presence of H-2^d was also performed on these sections; as acetone fixation abolished the PKH26-PCL fluorescence, an unfixed section was first viewed for the presence of PKH26-PCL, and the remainder were fixed. Sections were stained in a Sequenza (Shandon, Pittsburgh, PA) to reduce background staining. Slides were blocked sequentially with NMS, avidin D, and biotin blocking solutions (Vector Laboratories, Bretton, U.K.) before the addition of biotin conjugated mouse anti-mouse H-$2D^d$ mAb (PharMingen, San Diego, CA). Slides were washed, and ABComplex/AP (avidin/biotinylated alkaline phosphatase kit, Dako, High Wycombe, U.K.) and Vector Red alkaline phosphatase substrate kit (Vector Laboratories) were added before counterstaining with hematoxylin. Control slides were stained in the absence of the biotinylated anti-H-2^d Ab; tissue from H-$2^{k/d}$ mice acted as the positive controls and that from H-2^k mice acted as negative controls in each staining run. Formalin-fixed tissue was paraffin embedded, and cut sections were stained with hematoxylin and eosin. Cytospin (Shandon, Cytospin 2) preparations of peritoneal lavage cells were stained with Diff-Quik (Baxter, Thetford, U.K.) or for nonspecific esterase (Sigma Chemical Co.). Differential cell counts were performed, counting 400 cells/slide.

Immunofluorescence and flow cytometry

Cells were blocked with NMS at 4°C for 15 min before immunofluorescence. Both direct and indirect immunofluorescence were performed following standard protocols. For direct immunofluorescence the Abs used were FITC-conjugated mouse anti-mouse H-$2D^d$ monoclonal IgM Ab (PharMingen) and FITC-conjugated mouse anti-human CD15 IgM mAb (Dako) as the control, and FITC-conjugated mouse anti-mouse H-$2K^k$ monoclonal IgG2a Ab (PharMingen) and FITC-conjugated mouse anti-human T cell CD45RO IgG2a mAb (Dako) as the control. Indirect immunofluorescence was performed using F4/80 (HB 198 from American Type Culture Collection, Rockville, MD; IgG2b), M1/70 anti-CR3 (Serotec, Kidlington, U.K.; IgG2b) Abs to detect Mϕ, and anti-phytochrome (Mac 51 from European Collection of Animal Cell Cultures, Porton Down, U.K.) as the control (IgG2b) and was detected with the $F(ab')_2$ rabbit anti-rat IgG-FITC conjugate (Serotec). Cells from peritoneal lavagates were examined for the expression of PKH26-PCL (red fluorescence), H-2^d, H-2^k, or Mϕ surface markers using a Coulter EPICS II flow cytometer. Dual color immunofluorescence was used to assess anti-H-2^d and anti-H-2^k binding and concomitant PKH26-PCL labeling; Mϕ forward and side scatter characteristics were first determined by identification of Mϕ using F4/80 and M1/70. The gating parameters and immunofluorescence patterns were checked using pure Mϕ obtained by adherence. As shown in Figure 1 FITC anti-H-2^d and anti-H-2^k binding was expressed relative to isotype control Abs. PKH26-PCL positivity in Mϕ was expressed relative to unlabeled control Mϕ.

Cell culture in vitro

To determine the retention of PKH26-PCL label with time and the effect of labeling on viability, cells were cultured in Iscove's modified DMEM (Life Technologies) supplemented with 100 U of penicillin and streptomycin and 10% FCS in hydrophobic Teflon foils at 37°C in 5% CO_2; samples were examined daily for viability, PKH26-PCL expression, and Mϕ number. Formalin fixation was confirmed by cell adherence to tissue culture-treated plastic slides; live cells were strongly adherent, while fixed cells were completely nonadherent (not shown). To obtain very pure populations of inflammatory peritoneal Mϕ, peritoneal lavage cells were cultured on pre-warmed BHK matrix-coated plates in Iscove's DMEM (Life Technologies) for 1 h. Nonadherent cells were washed off; adherent cells were then lifted off by 5-min incubation with 5 mM EDTA on ice. These cells (>94% Mϕ by nonspecific esterase staining and indirect immunofluorescence) were used to confirm the flow cytometry parameters used. These adherence-purified cells were not used for adoptive cell transfer because the adherence may have altered their characteristics. Matrix-coated plates were prepared by growing BHK cells (BHK 21, clone 13: European Collection of Animal Cell Cultures) to confluence, then removing them with trypsin/EDTA (Sigma Chemical Co.).

Statistics

The percentages of PKH26-PCL-labeled, H-$2^{k/d}$, and phagocytosed donor cells in peritoneal lavagates were logarithmically transformed, normalizing the data and allowing calculation of geometric means and confidence intervals. Linearity in the decline of labeled cells was tested for by means of orthogonal polynomials. Regression analysis was employed to determine whether there was coincidence at 96 h after cell transfer of the curves for transfer of 15 and 30 million donor cells. The total numbers of Mϕ free in the peritoneal cavity at each time point for CB1 and C3HF/KAM mice were compared using ANOVA.

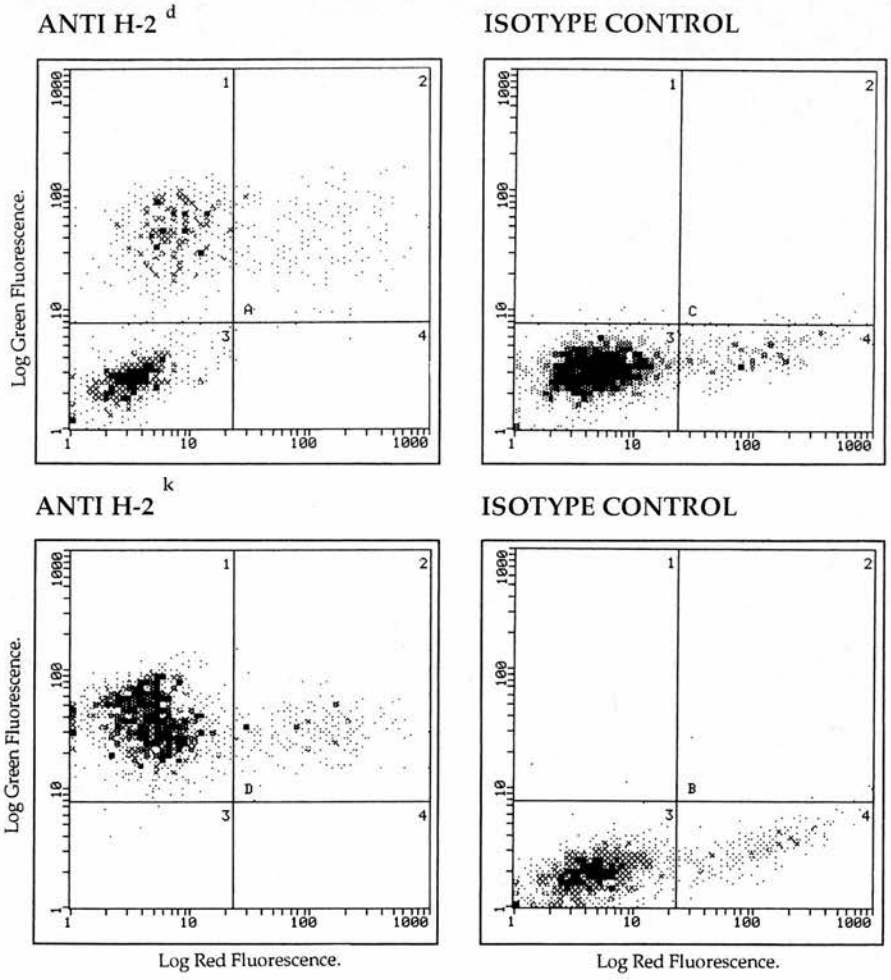

FIGURE 1. Representative experiment showing two-color flow cytometry of Mϕ from peritoneal lavage of a C3HF/KAM mouse (H-2^k) 48 h after adoptive transfer of live donor cells from a CB1 (H-$2^{d/k}$) mouse (approximately one-third of which were PKH26-PCL labeled). Green fluorescence (FITC-conjugated Abs) is shown on the *y*-axis; red fluorescence (PKH26-PCL) is shown on the *x*-axis. Binding of anti-H-2^d and anti-H-2^k Abs relative to their isotype controls was determined. Four regions are identified for each Ab using quad-stats: 1 = green positive, red negative; 2 = green positive, red positive; 3 = green negative, red negative; and 4 = green negative, red positive. PKH26-PCL red fluorescent-labeled cells were thus (anti-k region 2 − isotype control region 2), while H-2^d-positive cells were (anti-d regions 1 and 2 − isotype control regions 1 and 2). Phagocytosed cells were (anti-d region 4 − anti-k region 4).

Results

Time course of inflammatory response to TG: identification of resolution of inflammation

Both CB1 mice and C3HF/KAM mice had $<3 \times 10^6$ resident Mϕ in the peritoneal cavity before the induction of inflammation. Approximately 40% of these cells were Mϕ, demonstrated by differential counts and immunofluorescence; the bulk of the remainder were lymphocytes, in accordance with results previously published (30). TG induced a prompt PMN influx that declined after 2 days, while both the Mϕ number and relative percentage reached a peak on the fifth day after i.p. TG, with $19.6 \pm 1.8 \times 10^6$ (mean ± SD) cells recovered per mouse, >85% of which were Mϕ. As shown in Figure 2, both the total number of cells and the number of Mϕ steadily declined thereafter; thus, day 5 after TG was chosen as the optimum for cell transfer to follow Mϕ kinetics during resolution. There was no difference in the Mϕ kinetic response in CB1 or C3HF/KAM mice. A similar, although less pronounced, pattern was found using starch as the inflammatory agent, with $9.2 \pm 1.4 \times 10^6$ cells recovered on the fifth day after i.p. starch and >85% again being Mϕ. This declined significantly to $4.1 \pm 0.9 \times 10^6$ cells by day 10 ($p < 0.05$).

In vivo fluorescent cell labeling

At the concentration used, in the presence of inflammation, the fluorescent dye PKH26-PCL strongly labeled approximately one third (range, 27–42%) of the Mϕ population in vivo; the remaining cells were unlabeled (Fig. 3). This labeling pattern may reflect rapid uptake of the dye as the labeling percentage did not increase if the dye was left in situ for 1.5 or 18 h before lavage. Furthermore, supernatant from the lavage, after cell labeling, could not further label fresh cells in vitro, suggesting that the dye is rapidly taken up in the local region of the injection, rather than specifically labelling a subset of Mϕ. Moreover the characteristics of the labeled and unlabeled Mϕ were identical in regard to their expression of CR3, F4/80, H-2^d, H-2^k, cell size, adherence, and viability (data not shown). In vitro culture of labeled cells confirmed the retention of PKH26-PCL over the course of the experiment, with no difference in the viability of labeled or unlabeled cells. Formalin fixation did not affect this.

Adoptively transferred Mϕ numbers at the inflamed site decline rapidly

To follow the resolution kinetics of inflammatory Mϕ, 30×10^6 CB1 cells, all expressing H-$2^{k/d}$ and approximately one-third PKH26-PCL labeled (>95% viable; >85% Mϕ) were transferred into the peritoneal cavities of C3HF/KAM (H-2^k) recipients that were at the same stage of resolving inflammation. Figure 4 shows the recovery of labeled cells from peritoneal lavagates at different times after cell transfer. The percentage of H-$2^{k/d}$ cells and red fluorescent PKH26-PCL-labeled cells declined proportionally over 96 h effectively to zero. The ratio of PKH26-PCL-labeled cells to H-$2^{k/d}$ cells remained at approximately one-third over these 4 days, indicating that the labeled cells were not handled differently from the nonlabeled cells. There was little evidence for phagocytosis of

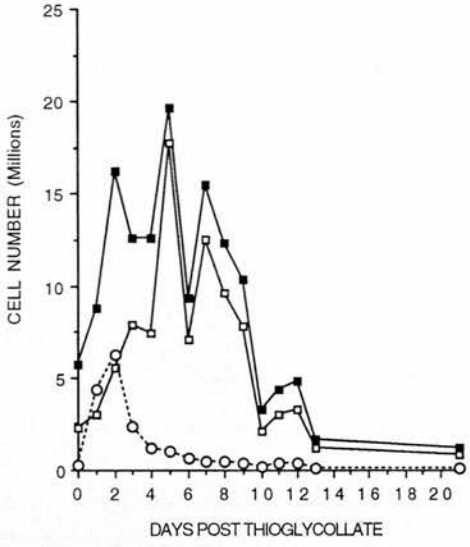

FIGURE 2. Inflammatory cell response to i.p. TG for CB1 mice. The total number of leukocytes (closed squares), the total number of Mϕ (open squares), and PMN (open circles) in peritoneal lavage with time are shown. Two milliliters of TG was injected i.p. on day 0, cells were recovered using 5 ml of PBS lavage. The total cell number was estimated by hemocytometer; >400 cells were counted. Mϕ and PMN number were obtained from relative percentages on duplicate cytospins. There was no difference in the percentage of Mϕ estimated using Diff-Quik staining, nonspecific esterase staining, or immunofluoresence with M1/70. The inflammatory response in C3HF/KAM mice was identical. Lymphocytes account for the remaining cells in differential counts (not shown). Each point represents the mean for at least four mice; all SD are within 10% of the means.

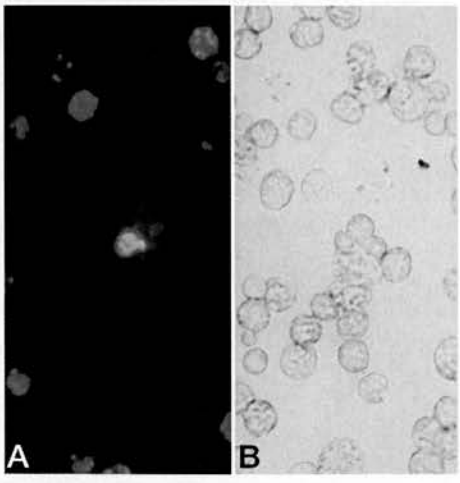

FIGURE 3. PKH26-PCL labeling of inflammatory Mϕ. Cytospin of peritoneal lavage cells was performed 5 days after i.p. TG and 1.5 h after i.p. PKH26-PCL labeling, shown by red fluorescent light (*A*) and the same field under light microscopy (*B*). In this particular field, less than one-third of the Mϕ are strongly red fluorescent labeled. Both fields were photographed at ×200 magnification.

transferred Mϕ in the peritoneal cavity during this time, with >90% of PKH26-PCL labeled Mϕ expressing donor H-$2^{k/d}$ rather than recipient H-2^{k} Ag. At 1 wk after adoptive cell transfer, the small percentage of red fluorescent cells remaining represented those that had phagocytosed labeled cells; all were H-2^{k} recipient Mϕ. Transfer of half the donor cell number did not alter the pattern of cell recovery; donor cells still took 96 h to completely disappear from the peritoneal lavage. Despite the transfer of a large number

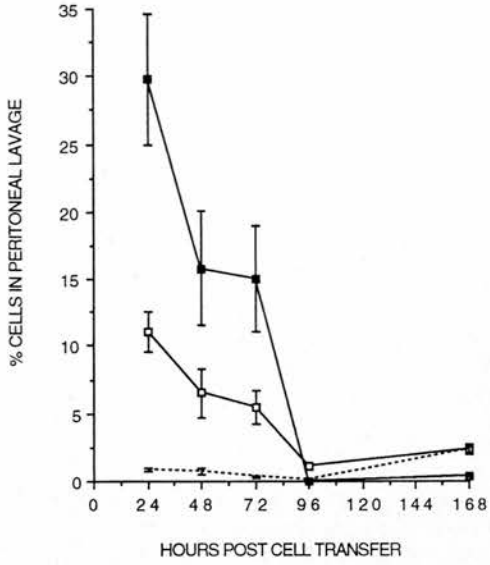

FIGURE 4. The percentage of donor (H-$2^{k/d}$) Mϕ recovered from the peritoneal cavity of recipient (H-2^{k}) mice with time after adoptive transfer of 30×10^6 live PKH26-PCL-labeled donor cells. H-$2^{k/d}$-positive cells (closed squares), PKH26-PCL positive cells (open squares), and phagocytosed cells (PKH26-PCL labeled cells expressing only recipient H-2^{k} surface Ag; dashed line) are shown. Each point represents the geometric mean for at least six mice, with 95% confidence intervals.

of Mϕ, the total number of Mϕ free in the peritoneal cavity declined steadily, with approximately 7×10^6 free cells 1 wk after adoptive transfer. Using starch as the inflammatory challenge in an identical set of experiments, a similar pattern of clearance was seen. The percentage of donor cells recovered declined from 25 ± 5% 24 h after semiallogeneic cell transfer to 1.1 ± 1.9% 1 wk after cell transfer ($p < 0.05$; $n = 3$). Again there was no evidence of local phagocytosis of donor Mϕ in the peritoneal cavity.

Adoptively transferred formalin-fixed Mϕ are rapidly phagocytosed at the inflamed site

To address the question of whether the decline in live adoptively transferred cells required active emigration and to confirm that the system was capable of detecting phagocytosis of donor cells, formalin-fixed donor Mϕ were transferred in a set of identical experiments. The time course for recovery of these fixed CB1 cells (>91% Mϕ) into C3HF/KAM recipients is shown in Figure 5. Virtually all the fixed cells were phagocytosed within 24 h; in contrast, <10% of the live cells were ingested during the first 96 h. Not only was the magnitude of the phagocytic signals grossly different between transfer of live cells and that of fixed cells, but the kinetics of live and fixed PKH26-PCL cells also differed significantly by 96 h after transfer, with live labeled donor cells exhibiting a linear decline to just above zero (1.5%) at 96 h, while fixed cells exhibited no discernible trend.

Semiallogeneic Mϕ kinetics are the same as those of syngeneic Mϕ

To investigate whether the presence of foreign Ags on the adoptively transferred cells altered the kinetics of Mϕ emigration over the time course investigated, PKH26-PCL-labeled Mϕ from C3HF/KAM mice were transferred into C3HF/KAM recipients in an otherwise identical model of inflammation. The detection of PKH26-PCL-labeled cells with time in peritoneal lavage for syngeneic and semiallogeneic cell transfer is shown in Figure 6; the

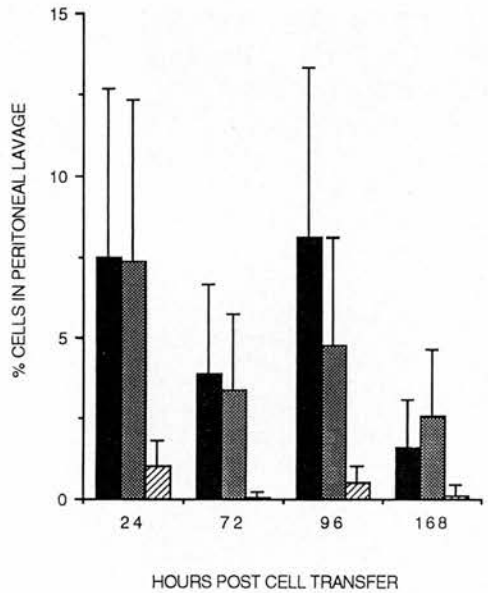

FIGURE 5. Recovery of formalin-fixed donor Mϕ from the peritoneal cavity of recipient mice with time after adoptive transfer of 30×10^6 formalin-fixed, PKH26-PCL labeled cells. PKH26-PCL-labeled cells (solid bar), phagocytosed cells (PKH26-PCL-labeled cells expressing only recipient H-2^k surface Ag; stippled bar), and nonphagocytosed H-$2^{k/d}$ cells (hatched bar) are shown. Each point represents the geometric mean for at least six mice, with 95% confidence intervals.

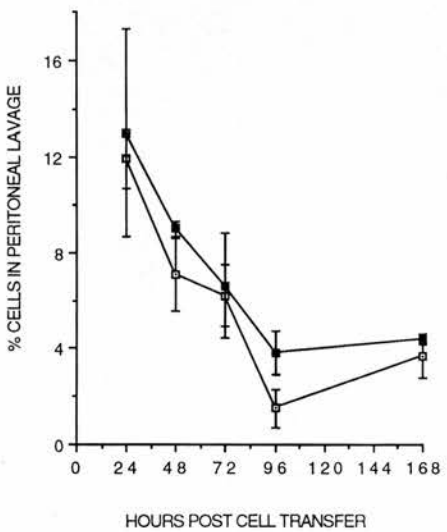

FIGURE 6. Comparison of syngeneic and semiallogeneic cell transfer. The percentage of PKH26-PCL-labeled donor Mϕ recovered from the peritoneal cavity of recipient mice with time after adoptive transfer of 30×10^6 cells was determined. Syngeneic cell transfer (C3HF/KAM into C3HF/KAM; closed squares) and semiallogeneic cell transfer (CB1 into C3HF/KAM; open squares) are shown. Each point represents the geometric mean of at least four mice, with 95% confidence intervals.

kinetics of the semiallogeneic cells closely mimic those of the syngeneic cells. Similar results were obtained for BALB/c as well as CB1 syngeneic cell transfers (not shown).

Mϕ emigrate from the inflammatory site to the draining LN

Since the number of live, adoptively transferred, Mϕ free in the peritoneal cavity declined rapidly, and we could not detect phagocytosis, we sought to identify the tissue fate of these cells.

Table I. *Tissue distribution of PKH26-PCL-labeled cells after adoptive cell transfer of in vivo-labeled live cells*[a]

Tissue	Hours Post-Cell Transfer					
	4	24	48	72	96	1 Wk
Lung	−	−	±	−	−	−
Heart		−	−	−	−	−
Kidney	−	−	−	−	−	−
Spleen	−	sp±	sp±	−	sp	++
Liver	−	−	sp±	−	−	++
Peritoneum	−	−	±	−	−	±
Omentum	++	++	+++	++	++	++
Lymph nodes						
Parathymic	−	++	++	+++	+++	+++
Para-aortic	−	−	±	±	±	−
Mesenteric	−	−	±	±	−	+

[a] Tissue distribution of PKH26-PCL label with time after transfer of 30×10^6 donor cells (>85% Mϕ) into peritoneal cavity of recipient mice at the same stage of resolving inflammation as the donor mice. Unfixed 3-μm frozen sections viewed under red fluorescence. "Peritoneum" represented peritoneal lining tissue adjacent to anterior abdominal wall. Semiquantitative assessment of PHK26-PCL-labeled cells thus: − = no PKH26-PCL-labeled cells per section; ± = 1–3; + = 4–8; ++ = 8–15; +++ = >15 PKH26-PCL-labeled cells per section. sp = specks, not whole cells. Results are means for eight mice per time point (four mice for 4-h time point).

Frozen sections of peritoneum including omentum, a range of LN, liver, spleen, kidney, lung, and heart were examined for PKH26-PCL-positive cells. Table I shows the tissue distribution of PKH26-PCL-labeled cells with time. There was no evidence of widespread adherence of transferred Mϕ to the peritoneal lining, with adherent labeled cells confined only to collections on the omentum. These collections of varying numbers of PKH26-PCL-positive cells, ranging from a few cells to several dozen cells, appeared to represent "milky spots" on the omentum (32). These aggregates did not change in size or frequency with time, being as numerous 4 h after transfer as they were 96 h after transfer. A large number of labeled Mϕ was detected specifically in the draining LN of the peritoneum, the parathymic LN (Fig. 7). The appearance of labeled cells did not occur before 4 h after cell transfer; thereafter, they were detected for 1 wk. These cells were initially located in the subcapsular region (Fig. 7*A*), but by 48 h after cell transfer, many labeled cells were also detected deep within the node tissue (Fig. 7*B*), surrounded by lymphocytes. Despite quantification difficulties, it was clear that there were more labeled cells in the parathymic LN at the later time points (72 h, 96 h, and 1 wk) than at the earlier points (4, 24, and 48 h) after cell transfer. PKH26-PCL label was detected in the liver and spleen 1 wk after cell transfer, but was often very faint.

Interestingly, the tissue distribution of PKH26-PCL with transfer of fixed Mϕ was remarkably similar to that observed following transfer of live cells (data not shown). Again, there were collections of PKH26-PCL-positive cells on the omentum, commensurate with milky spots, and the parathymic LN were again specifically and strongly labeled with PKH26-PCL-positive cells, label was detected in the liver and spleen at 1 wk. No label was seen in the lung, heart, or kidney at any time after transfer of live or formalin-fixed donor Mϕ, and only occasional labeled cells were seen in the para-aortic and mesenteric LN. This indicated that recipient cells with phagocytosed donor Mϕ were capable of emigration, and this followed a similar pattern to that seen with transfer of live donor Mϕ.

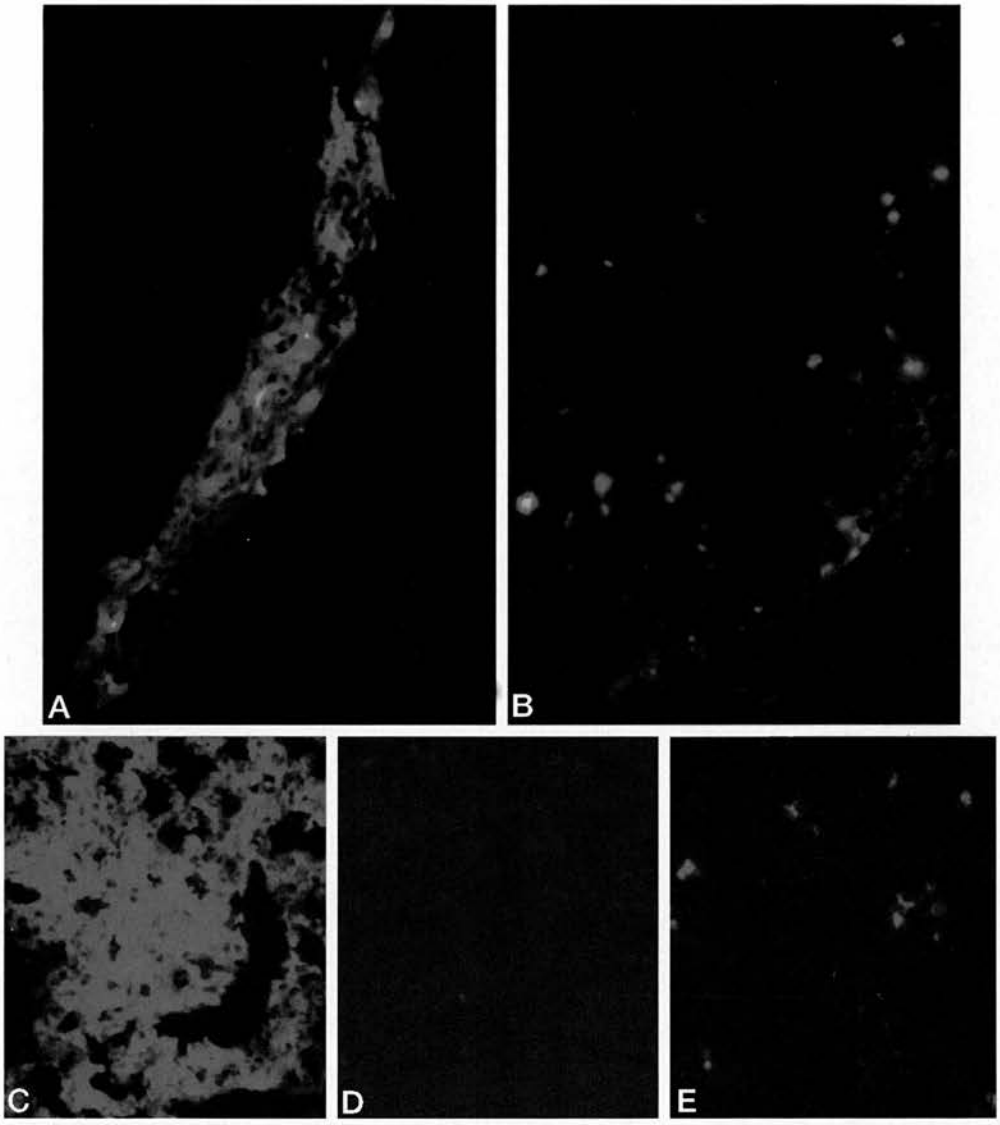

FIGURE 7. Donor Mϕ migrate intact to the draining LN. *A*, Photograph under red fluorescence of parathymic LN frozen section from recipient mouse 24 h after adoptive transfer of live donor PKH26-PCL-labeled Mϕ, showing strongly fluorescent cells especially in the subcapsular region. *B*, Parathymic LN from recipient mouse 48 h after live adoptive cell transfer, PKH26-PCL-labeled cells are now widely scattered throughout the node. *C*, Positive control for H-2^d immunostaining; CB1 parathymic node stained with biotinylated anti-H-2^d, using avidin/biotin-alkaline phosphatase and Vector Red alkaline phosphatase substrate, taken under red fluorescence, demonstrating H-2^d expression on all the cells. *D*, Parathymic node from C3HF/KAM mouse as a negative control stained as described for *C*, showing no H-2^d expression. *E*, H-2^d staining of parathymic node of recipient C3HF/KAM mouse 1 wk after transfer of live H-$2^{k/d}$ donor Mϕ, now showing a number of positive donor cells within the node. All photographs were taken at ×200 magnification.

Adoptively transferred live Mϕ are not phagocytosed before emigration

To confirm that adoptively transferred live Mϕ were indeed emigrating to the parathymic LN and not being passively carried there after phagocytosis by recipient Mϕ, frozen sections were examined for the presence of H-2^d. As processing for anti-H-2^d staining destroyed PKH26-PCL fluorescence, no double staining was possible. However, serial sectioning demonstrated that after live cell transfer, H-2^d staining was always and only present when there was PKH26-PCL staining on adjacent sections. Positive staining for H-2^d in the parathymic LN confirmed that it was the live donor Mϕ themselves that had emigrated from the inflamed site (Fig. 7*E*). In contrast, there was no H-2^d staining in the parathymic LN after the return of fixed Mϕ, despite the strong PKH26-PCL fluorescence. Thus, live donor Mϕ emigrated intact to the draining LN, while fixed Mϕ were phagocytosed within the peritoneal cavity; a proportion of the phagocytosing Mϕ then emigrated to the draining LN, where they could be detected by the presence of the ingested red fluorescent label. Staining for the presence of H-2^d in the spleen demonstrated only occasional intact donor cells, suggesting that the bulk of the PKH26-PCL fluorescence detected at 1 wk did not represent further migration of cells from the LN.

Parathymic LN histology

Macroscopically at early time points after cell transfer the LN were grossly enlarged, returning to normal size by 1 wk, as confirmed by the reduction in weight of the LN from 67 ± 6.8 mg at the time of cell transfer to 11.25 ± 10.7 mg (mean ± SD) 1 wk later. The inflammatory reaction in these LN was consistent with a resolving granulomatous response, with several multinucleate giant cells being detected per section. Over the first 3 days after cell transfer, the subcapsular space was packed with intensely vacuolated Mϕ; the

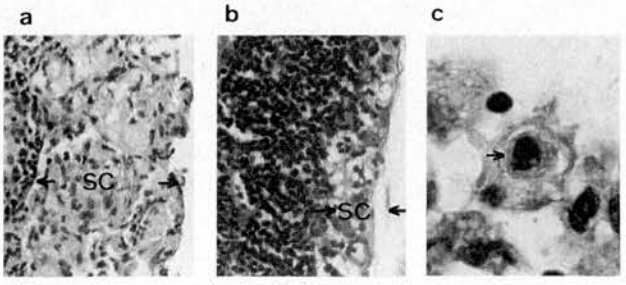

FIGURE 8. The subcapsular space Mϕ predominance accompanying inflammation diminishes with resolution. Hematoxylin and eosin (H&E)-stained sections of parathymic LN 6 days after i.p. TG during acute inflammation, demonstrating the grossly enlarged subcapsular space (SC) full of vacuolated Mϕ (*a*), and 12 days after i.p. TG with resolution of inflammation the subcapsular space returns to normal (*b*). Both sections taken at ×100 magnification. *c*, Paraffin section stained with H&E of parathymic LN from mouse that had received 30×10^6 formalin-fixed adoptively transferred Mϕ i.p. 48 h before showing a phagocytosed Mϕ (arrow) lying within a vacuole.

number of Mϕ and their apparent size then declined steadily (Fig. 8, *a* and *b*). Transfer of phagocytosed fixed cells from the peritoneal cavity to the parathymic LN was suggested on hematoxylin- and eosin-stained sections by the appearance of a number of Mϕ bearing identifiable Mϕ within phagocytic vacuoles (Fig. 8*c*).

Discussion

Although much work has been undertaken on constitutive resident Mϕ turnover in the noninflamed steady state peritoneum, this is the first detailed study of the fate of the inflammatory Mϕ during the resolution of inflammation using adoptive transfer of labeled Mϕ to quantify the cellular kinetics. By contrast with the PMN or mesangial cell, there is clear evidence that inflammatory Mϕ do not meet their fate locally by apoptosis and phagocytosis, but that they emigrate from the peritoneum to the draining LN. It has long been known that Mϕ are able to migrate to lymph nodes (7, 33–35), but in this study we demonstrate that this is the major mechanism for the removal of inflammatory Mϕ from the peritoneum during the resolution of inflammation and that the time course for this clearance is rapid. The emigration process takes longer than 4 h, but most adoptively transferred Mϕ had been cleared within 96 h. Upon reaching the LN, the cells become localized initially to the subcapsular space; by 48 h after cell transfer, however, many cells were detected deeper within the LN, surrounded by lymphocytes. The emigration of the inflammatory Mϕ occurs specifically to the draining LN; there is little evidence of migration of donor Mϕ to other LN. Although PKH26-PCL label could be detected in the liver and spleen 1 wk after transfer, this was accompanied by the detection of very few H-2^d-positive cells. Hence, the draining node apparently represents the "final resting place" for the bulk of the inflammatory Mϕ. Thus, these data suggest that monocyte/Mϕ undergo a two-stage migration process. Initially as monocytes they emigrate from the blood to the inflamed site where they fulfill their inflammatory and scavenging roles. Thereafter, a matter of days later in this particular model of resolving inflammation, the inflammatory Mϕ migrate to the draining LN, where they would be well positioned to contribute to the generation of an immune response.

Previous studies with tritiated thymidine labeling of dividing cells have given an indication of the local rates of cell turnover in certain inflammatory events (36, 37). Loss of label from the inflammatory site would not, however, have indicated whether this was due to cell death or emigration. Indeed, should there have been significant reutilization of radiolabel subsequent to the phagocytosis of dead labeled cells, the cell life-span would have appeared falsely prolonged. Thus, if significant reutilization of radiolabel occurs, detection of radioactivity at local or distant sites would not necessarily have indicated the fate of the adoptively transferred labeled cells themselves. Moreover, as Mϕ are capable of significant proliferation at the site of inflammation (17, 38, 39), uncertainty about the relative contributions of cell proliferation and cell influx causes further interpretation difficulties. Our data using this dual labeling technique showed that the decline in Mϕ recovery at the inflamed site was not due to local phagocytosis and that the cells detected distantly in the parathymic LN were indeed intact donor cells. The reduction in recovery of donor cells in the peritoneal cavity with time was not due to widespread adherence to the peritoneum. Although Mϕ adhered to the omentum in milky spot aggregates, these did not increase in number or size over the time course studied. Hence, increased adherence alone was unlikely to account for the observed decrease in donor cell recovery. After adoptive transfer of live Mϕ, there was little evidence of their phagocytosis; the PKH26-PCL-labeled cells recovered expressed the donor H-$2^{k/d}$ rather than the recipient H-2^k surface Ag. Nor was the decrease in the percent donor cells recovered due to a dilution effect from the influx of unlabeled cells, since the total number of lavagable Mϕ also declined, consistent with resolving inflammation. This, in conjunction with the tissue data demonstrating the accumulation of labeled cells within the draining LN, suggested that emigration of the inflammatory Mϕ from the peritoneum was the main mechanism responsible for the clearance of these cells.

The reduction in recovery of donor cells from the peritoneal cavities of recipient mice was not due to a reaction to the presence of foreign Ag (H-2^d), as demonstrated by the similar kinetics for semiallogeneic and syngeneic cells after adoptive cell transfer. Other workers using allogeneic or semiallogeneic cell transfer have also found little difference in cell kinetics over the first few days compared with syngeneic transfers (40). Consistent with this interpretation, there was no evidence for a secondary inflammatory response histologically or in the peritoneal lavagates after adoptive cell transfer.

PKH26-PCL-labeled cells were not handled differently from unlabeled cells; the decline in H-$2^{k/d}$ cells was proportional to that in PKH26-PCL-labeled cells, and both detection methods indicated a steady drop in the number of adoptively transferred cells free in the peritoneum. The decline in the percentage of PKH26-PCL-positive cells recovered was not due to loss of the label. This was demonstrated both by in vitro culture and in vivo tissue section results, and other groups have confirmed that although labeling intensity declines slowly with time, labeled Mϕ can still be distinguished from unlabeled Mϕ for up to 7 wk (12, 41).

In contrast to the results for live cell transfer, transferred formalin-fixed cells were phagocytosed quickly and efficiently, with very few remaining free by 24 h, suggesting efficient clearance of dead or effete cells by inflammatory Mϕ. This observation also confirmed that TG-elicited Mϕ were effective phagocytes, despite reported differences in their bactericidal capacity compared with Mϕ induced by other inflammatory stimuli (37). The experiments using starch as the inflammatory stimulus showed that not only was the time course of Mϕ accumulation similar, but the clearance pattern appeared almost exactly the same as that for TG-elicited Mϕ. This suggests that the results with TG may be broadly relevant to the resolution of acute inflammation.

The kinetics of removal of the phagocytosed fixed transferred cells was slower than that of the live transferred cells. There was

clear evidence that the recipient Mϕ remained functionally intact after phagocytosing formalin-fixed donor cells, since they were able to emigrate to the draining LN. Thus, the delay in clearance of labeled cells was unlikely to be due to the formalin-fixed donor cells being toxic to the engulfing cells. The possibility that a subpopulation of Mϕ with slower emigration capability was involved in phagocytosis of fixed cells was also unlikely, since the populations of donor and recipient cells were elicited in the same way. An alternative and plausible explanation is that phagocytosis of the deliberately killed, transferred Mϕ activated the phagocytosing Mϕ in a manner that prolonged their presence at the inflamed site. We have previously shown in vitro (22, 42) that Mϕ uptake of necrotic, but not apoptotic, neutrophils and eosinophils caused Mϕ activation and secretion of thromboxane B_2 and granulocyte-macrophage CSF.

The rate of turnover for resident peritoneal Mϕ in the steady state reported in the literature differs markedly from the data presented here for inflammatory Mϕ during the resolution of sterile inflammation. Previous work on the kinetics of resident peritoneal Mϕ suggested a mean turnover time of 14.9 days (13). Adoptive transfer of radiolabeled resident peritoneal Mϕ indicated that even 2 wk after cell transfer, more than half the labeled cells could be recovered in the peritoneal lavagate (10). More recent experiments using fluorescent-labeled resident peritoneal Mϕ are somewhat contradictory; one group using an in vitro labeling technique reported a rapid decline in cell retrieval (8), while others using in vivo labeling again demonstrated a prolonged half-life of several weeks or more for the resident peritoneal Mϕ population (12, 14).

The tissue distribution of PKH26-PCL-labeled cells after leaving the inflammatory site is in accordance with that reported previously for resident Mϕ emigration (8, 10). The lymphatics draining the peritoneum arise from the subdiaphragmatic surface, entering ducts running with the internal mammary vessels to reach the anterior mediastinal LN. Efferent lymphatics from these usually run to the right lymphatic duct, with some cross-communication to the thoracic duct (43). Elegant electron microscopy studies have confirmed that stomata exist between the mesothelial cells overlying lymphatic spaces, and these widen during inflammation, allowing for cell emigration (32, 44). Within the parathymic LN themselves, the PKH26-PCL-labeled cells were initially distributed in the subcapsular region, consistent with the histologic findings. However, 48 h or more after cell transfer, many labeled cells were also observed deep in the node. This, again, is in accordance with previous work on resident peritoneal Mϕ emigration; cells were detectable in the outer cortex and medullary cords of LN (8, 31). Mϕ migration to LN has been demonstrated in other systems (33–35), but quantification of rates of emigration and cell death has not been performed. The distribution pattern that we have demonstrated for inflammatory Mϕ in the draining LN would allow for Ag presentation to lymphocytes. Since Mϕ are widely recognized as "professional" APC, their emigration to the LN, where interaction with immunocompetent cells is maximized, may well have functional implications beyond that of simple cell clearance.

It is interesting to review this work in the light of what is known of the fate of the PMN with resolving inflammation. The PMN is a short-lived cell that dies at the site of inflammation, where it is phagocytosed by the inflammatory Mϕ. Similarly, the mesangial cell, a myelofibroblast-like cell with phagocytic potential in the kidney, has been shown in experimental inflammation to die in significant numbers by apoptosis and to be cleared by local phagocytosis (2). In contrast, the inflammatory Mϕ is a potentially long-lived cell with a much broader repertoire of functions, including Ag presentation. It does not seem to die locally at the inflamed site, but, rather, after migration into the inflamed site as the monocyte, it is seen later to emigrate nonrandomly to the draining LN, a situation more analogous with that of lymphocyte trafficking or Langerhans cell migration. The clearance of inflammatory Mϕ from the site of acute inflammation is rapid, with large numbers of Mϕ emigrating daily during resolution. This two-stage migration process in the life-span of the inflammatory Mϕ is well suited to its functions, allowing for phagocytosis of materials at the inflamed site and Ag presentation in the LN, and thus may have implications for the initiation and amplification of immune responses against agents derived from the inflamed site.

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Hypoxia prolongs neutrophil survival in vitro

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Abstract Neutrophil apoptosis represents a major mechanism involved in the resolution of inflammation. Since hypoxia induces apoptosis in several cell lines and is of particular relevance in many disease states, we studied the effect of oxygen concentration on neutrophil survival in vitro. Hypoxia caused a dramatic decrease in neutrophil apoptosis (% apoptosis 20 h: 78.7 ± 2.2% in 21% O_2, 61.4 ± 6.5% in 2.5% O_2, 23.1 ± 3.2% in 0% O_2, $n = 5$). This was additive to the effect of GM-CSF (50 U/ml), not associated with induction of *bcl*-2 expression, and was not mimicked by methionine (5 mM), superoxide dismutase (200 μg/ml) or Trolox (10 mM) but was mimicked by catalase (250 μg/ml). Hence, hypoxia has a *bcl*-2-independent effect on neutrophil apoptosis that may adversely affect the clearance of these cells from an inflammatory focus.

Key words: Hypoxia; Neutrophil; Apoptosis; Bcl-2; Oxidant; Antioxidant

1. Introduction

During a bacterial invasion the body mounts an immune response involving recruitment of inflammatory cells into the area of infection [1]. One of the principal cells involved in this response is the neutrophil, which, when stimulated by a pathogen, degranulates and undergoes respiratory burst activity, to release a variety of histotoxic enzymes and short-lived reactive oxygen species (ROS) [2]. Many of the antiproteinases that protect the host against neutrophil-mediated injury [3] are inactivated by oxidation, and hence local oxidant-antioxidant balance appears to be a critical factor determining the extent of tissue damage [4]. The many deleterious contents of neutrophils necessitate that for complete resolution of inflammation these cells should be removed in a way that limits cell activation, and neutrophil apoptosis represents an important mechanism wherby this may be achieved [5,6,7].

When neutrophils die by apoptosis they retain their granule contents but lose chemotactic and secretory responsiveness [8] and thereafter are recognised and phagocytosed intact by macrophages [9] which fail to release pro-inflammatory mediators in response to this interaction [10]. Although neutrophils undergo constitutive apoptosis in vitro [11], their survival may be increased by the addition of certain cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and bacterial products such as lipopolysaccharide (LPS) [12,13]. However, the intracellular signalling pathways that regulate neutrophil survival are unclear.

Studies on the nematode, *C. elegans*, have shown that individual cell survival is signalled by the gene, *ced*-9 [14]. The mammalian homologue of the Ced-9 protein is Bcl-2 [15], which is capable of causing survival of a number of cell-lines and B-lymphocytes [16,17], an effect potentially mediated by the ability of Bcl-2 to inhibit oxidant-mediated cell damage [18,19]. This hypothesis arose from studies in which oxidant treatment of FL5.12 cells, an IL-3 dependent pro-B lymphocyte cell-line, caused an increase in apoptosis that could be counteracted by over-expression of *bcl*-2 [18], and studies in GT1–7 neural cells where *bcl*-2 expression prevented death induced by glutathione depletion [19]. Bcl-2 did not prevent free radical production but did appear to block the damaging effects of these agents. In contrast, Jacobson and Raff [20], and Shimizu et al. [21], have shown that the protective effects of Bcl-2 are observed even under extreme hypoxic conditions where the generation of ROS is negligible. These latter studies also demonstrate that hypoxia alone can induce apoptosis in certain cells [20,21].

To date, all the studies examining the potential involvement of reactive oxygen intermediates in regulating apoptosis have been undertaken in cell lines to allow manipulation of *bcl*-2 levels. This is the first report to examine the effects of hypoxia on apoptosis in the neutrophil, a terminally differentiated cell that lacks detectable Bcl-2 [22]. In contrast to the above studies, we demonstrate a profound inhibition of neutrophil apoptosis by hypoxia. This effect of hypoxia on neutrophils may be of considerable relevance to conditions in vivo where the oxidant potential at sites of inflammatory disease may be extremely low [23,24].

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals and enzymes were purchased from Sigma (Dorset, UK), and all antibodies obtained from Dako (Bucks., UK). The plasticware was supplied by Becton Dickinson (Leics., UK) or Nunc (Paisley, UK), and the culture media were obtained from Life Technologies (Paisley, UK). Percoll and dextran were purchased from Pharmacia (Milton Keynes, UK), and GM-CSF (specific activity 1.25×10^7 U/mg) from Genzyme Diagnostics (Kent, UK). Trolox (6-hydroxy-2,3,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Aldrich (Dorset, UK).

2.2. Isolation of neutrophils

Peripheral venous blood was collected from healthy volunteers and placed in citrated polypropylene tubes (0.38% (v/v) final concentration

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Abbreviations: ROS, reactive oxygen species; GM-CSF, granulocyte-macrophage colony-stimulating factor; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LPS, lipopolysaccharide; SOD, superoxide dismutase.

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of sodium citrate solution). The whole blood was centrifuged ($300 \times g$) for 20 min and the plasma removed. The leukocyte-rich fraction was separated from the erythrocytes in the remaining portion by dextran sedimentation [25]. Neutrophils in the leukocyte-rich fraction were separated from mononuclear cells using a discontinuous plasma/Percoll gradient and collected from the 42%/51% plasma/Percoll interface. The isolated neutrophils were >95% pure (<0.1% mononuclear cells) and >99% viable as determined by Trypan-blue dye exclusion. Previous studies have demonstrated that neutrophils isolated by this method are non-activated since they display <5% basal shape change, minimal basal superoxide or lysozyme generation and, unlike cells prepared using Ficoll-Hypaque gradients, exhibit normal chemotactic responses to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) [25].

2.3. Neutrophil culture

Freshly harvested neutrophils were suspended at a density of 5×10^6/ml in Iscove's DMEM supplemented with 10% autologous serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Neutrophils (6.75×10^5) were cultured in a final volume of 150 μl in flat-bottomed 96-well polypropylene flexiwell inserts. The plates were placed in sealed air-tight plastic boxes that were flushed through for 15 min with gas mixtures containing either 2.5% O_2/5% CO_2 or 21% O_2/5% CO_2 (control), with the balance made up with nitrogen. The boxes were then incubated at 37°C for the time periods indicated. More extreme hypoxic conditions were achieved by placing cultured neutrophils in an MK3 anaerobic incubator (0% O_2, Don Whitley Scientific Ltd., Yorkshire, UK) fitted with a palladium catalyst. The level and stability of pO_2 achieved using the 2.5% O_2 mixture was measured using a blood-gas analyser (model ABL-330 Radiometer, Copenhagen, Denmark). The pO_2 values obtained were 8.69 ± 0.73 kPa, 8.6 ± 0.32 kPa and 9.55 ± 1.66 kPa after 0, 6 and 20 h, respectively, in culture in 2.5% O_2. Equilibrium of the pO_2 and pCO_2 in the medium took less than 30 min with the boxes (2.5% O_2) and less than 1 h with the anaerobic incubator (0% O_2). To assess whether the effects of hypoxia influenced the known ability of GM-CSF to prolong neutrophil survival, cells were also incubated under hypoxic or normoxic conditions in the presence of absence of GM-CSF (50U/ml).

2.4. Morphological assessment of apoptosis

At the stated time points, neutrophils were gently resuspended, harvested and cytocentrifuged. The resulting slides were fixed and stained with Diff-Quick. Cell recovery and viability by Trypan-blue dye exclusion were measured in parallel. Cell morphology was examined under a 100× objective and apoptotic cells defined as cells containing darkly stained pyknotic nuclei [26]. Triplicate slides were prepared for each condition and at least 500 neutrophils counted per slide in random fields.

2.5. Treatment with oxidants and antioxidants

Freshly isolated neutrophils were suspended at a density of 5×10^6/ml in Iscove's DMEM supplemented with 10% autologous serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) and dispensed into flat-bottomed 96-well polypropylene flexiwell inserts. The neutrophils were treated with 200 μg/ml bovine erythrocyte SOD (750 U/ml), 250 μg/ml bovine liver catalase (4750 U/ml), 200 μg/ml SOD and 250 μg/ml catalase or 5 mM methionine. Control, untreated cells were also prepared. The cells were cultured for 6 or 20 h at 37°C under normoxic (5% CO_2:air) conditions, after which cytocentrifuge preparations were made and apoptosis assessed morphologically.

Assessment of the effect of hydrogen peroxide on the rate of neutrophil apoptosis was studied by treating freshly isolated neutrophils (1×10^6/ml of Iscove's DMEM supplemented with 2% autologous serum, 100 U/ml penicillin and 100 μg/ml streptomycin) with hydrogen peroxide (0.1–5 mM) in the presence or absence of the soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 10 mM). Control, untreated cells, and cells treated with antioxidant alone were also examined. The cells were cultured for 8 h at 37°C under normoxic (5% CO_2:air) conditions, after which cytocentrifuge preparations were made and apoptosis assessed morphologically.

2.6. Immunocytochemistry

Cultured cells (1×10^5) were transferred into round-bottomed 96-well polypropylene, flexiwell inserts. The cells were simultaneously fixed and permeabilised with 50 μl 0.01% (w/v) L-α-lysophosphatidylcholine in PBS containing 3.7% (v/v) formaldehyde for 15 min at 37°C [27]. The cells were then washed with PBS containing 0.5% (w/v) BSA and incubated (4°C) for 1 h with a saturating concentration of a mouse monoclonal antibody raised against human Bcl-2 protein. As a positive control, parallel samples were stained with MAC 387, a mouse monoclonal antibody which recognises p8,14, an intracellular calcium binding protein complex found in neutrophils. The amount of primary antibody binding was detected using a FITC-conjugated goat anti-mouse polyclonal antibody. Negative control cells were stained with second layer antibody alone. The samples were analysed using an EPICS profile II (Coulter Electronics, Luton, UK).

2.7. Statistics

All data are presented as mean ± S.E.M. for (n) separate experiments. Values were compared using the Student's t-test for paired data with $P < 0.05$ considered to be significant.

3. Results and discussion

Incubation of neutrophils under hypoxic conditions resulted in a dramatic inhibition of apoptosis (Fig. 1a) to the extent that cells incubated for 44 h in 0% O_2 were >95% viable and <25% apoptotic compared to nearly 100% apoptosis/ necrosis under normoxic (21% O_2) conditions (Fig. 1b). These findings are in complete contrast to those of Jacobson and Raff [20] and Shimizu et al. [21] who have shown that hypoxia induces apoptosis in SV-40 transformed human fibroblasts (that lacked mitochondria) and a rat pheochromacytoma cell line, respectively. A similar induction of apoptosis by hypoxia has been reported in WEHI 7.1 T-lymphoma cells [28], adenocarcinoma HT29 cells [29] and superior cervical ganglia cells [30]. In the studies of Jacobson and Raff [20] and Shimizu et al. [21], apoptosis could be inhibited under both normoxic and hypoxic conditions by transfected *bcl*-2. Since we were unable to detect any Bcl-2 protein in neutrophils aged under either hypoxic or normoxic conditions (Table 1), it is unlikely that the enhanced survival of neutrophils under hypoxic conditions was *bcl*-2-mediated.

We have previously demonstrated that cytokine growth factors such as GM-CSF are capable of extending the life of the neutrophil [31], and hence we investigated whether the effects of GM-CSF and hypoxia were mediated by a common mechanism. In fact, GM-CSF- and hypoxia-mediated survival of neutrophils were additive (Fig. 2), suggesting that the hypoxia and GM-CSF effects are independent.

To examine whether the effect of hypoxia was mediated by inhibition of ROS generation, we investigated whether exogenous addition of antioxidant enzymes and methionine, an essential thiol containing amino acid, would mimic the hypoxic

Table 1
Effect of hypoxic culture on Bcl-2 in neutrophils

% oxygen	Amount of detectable Bcl-2 (Relative Mean Fluorescence)	
	−GMCSF	+GMCSF
0%	1.14 ± 0.14	1.01 ± 0.24
21%	1.09 ± 0.12	1.01 ± 0.014

Neutrophils were incubated in the presence or absence of GMCSF (50 U/ml) for 20 h in atmospheres containing 0% or 21% O_2. The amount of Bcl-2 in the cells was measured as detailed in section 2 and expressed as mean fluorescence relative to negative control. In all cases, Bcl-2 could not be detected in the neutrophils. HL60 cells, which are known to express Bcl-2, were used as a positive control (relative mean fluorescence = 4.43). MAC 387, which recognises an intracellular antigen in neutrophils, was used confirm the efficient permeabilisation of the neutrophils (relative mean fluorescence > 50).

effect. These agents had no effect on cell viability after 20 h of culture as measured by Trypan-blue exclusion which supports a previous study [32]. Methionine and SOD did not influence the rate of neutrophil apoptosis (Fig. 3) suggesting that hypochlorous and superoxide radicals do not play a significant role in regulating neutrophil apoptosis. This supports a previous a study which demonstrated that SOD was ineffective in preventing cell death in human lung fetal fibroblasts [33]. In contrast, catalase, both alone, and in combination with SOD, caused a significant inhibition of neutrophil apoptosis (Fig. 3). It is probable, however, that this effect of catalase is independent of its effects on hydrogen peroxide levels since a similar anti-apoptotic effect of catalase has been demonstrated in WEHI 231 cells [34] and catalase has now been identified as the anti-apoptotic factor present in the conditioned medium of CCRF-CEM T-cell leukaemia cells [35]. This conclusion is supported by the lack of effect of SOD and Trolox. Of interest, we also observed that the endogenous SOD and catalase levels in neutrophils measured by standard methods [36,37], was significantly increased under hypoxic culture conditions (SOD activity after 20 h culture in 21% O_2 was 10.05 ± 0.287 U/mg protein and in 0% O_2 was 18.68 ± 3.32 U/mg protein ($P < 0.05$). Catalase activity after 20 h culture in 21% O_2 was 125 ± 13.83 U/mg protein and in 0% O_2 was 263 ± 32.71 U/protein ($P < 0.05$)). It is likely that this induction of antioxidant enzymes represents a stress response to the hypoxia since Das et al. have shown that Mn SOD and catalase are two of a number of stress-related genes up-regulated in cardiac biopsy tissue following repeated ischaemia [38]. Whether the anti-apoptotic effect of hypoxia relates to the induction of other stress-related proteins or changes in poly-ADP-ribose transferase activity and hence ATP levels [39,40], lipid oxidation [41], regulation of NFκB activation [42], or some alternative mechanism, requires further investigation.

Having shown that neutrophil survival in vitro can be prolonged by hypoxia where the cells' capacity to generate ROS, at least in 0% O_2, is severely diminished [18] we investigated the direct effect of ROS on the rate of neutrophil apoptosis. Hydro-

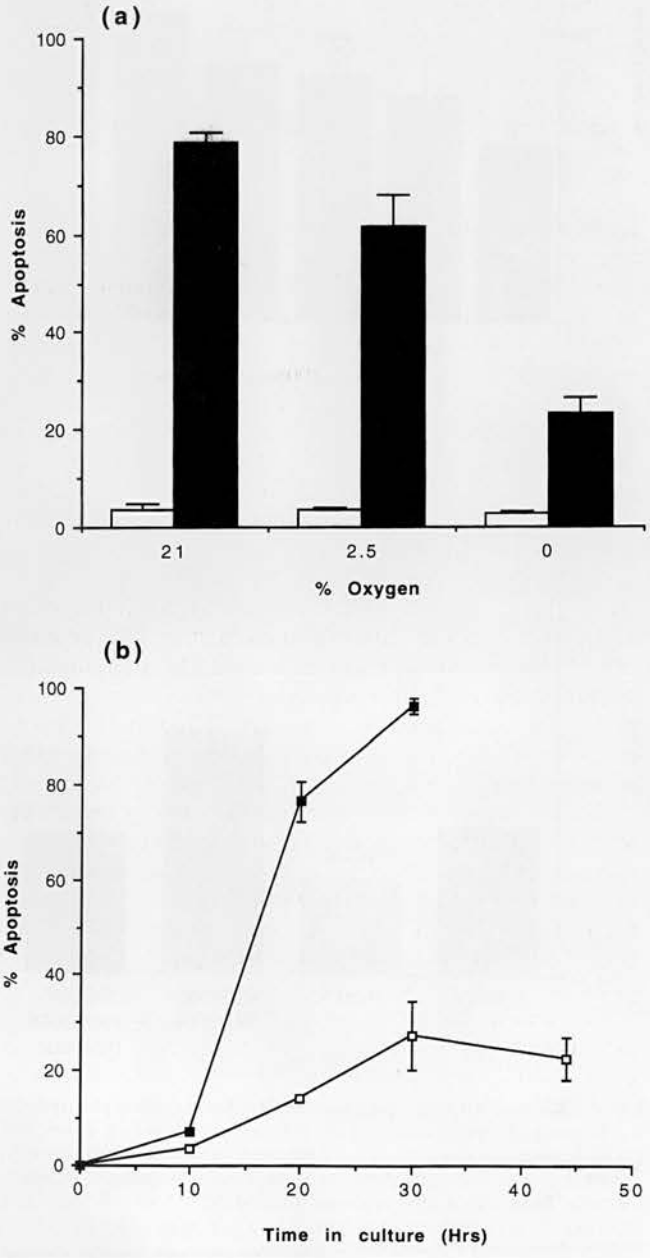

Fig. 1. Effect of hypoxia on neutrophil apoptosis. Neutrophils were incubated in atmospheres containing either 21%, 2.5% or 0% O_2 for 6 hrs (open bars) or 20 hrs (closed bars) in (a) or 21% (closed symbols) and 0% (open symbols) in (b). The percentage apoptosis was assessed morphologically as detailed in section 2 at the time points indicated. Data in (a) represent mean ± S.E.M. of $n = 5$ separate experiments, each performed in triplicate. In (b), culture of cells for 44 h in the presence of 21% O_2 caused a major increase in the percentage necrotic cells (as assessed by Trypan-blue dye exclusion) and therefore it was not possible to determine the percentage of apoptotic cells at this time point. The data in (b) represent mean ± S.E.M. of triplicate incubations from a single representative experiment.

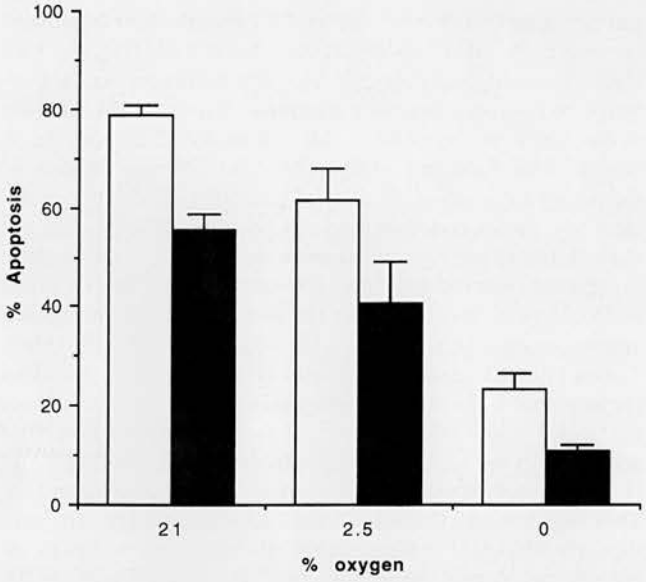

Fig. 2. Modulation of the GMCSF survival effect in neutrophils by hypoxia. Neutrophils were incubated with GMCSF (50 U/ml) for 20 h in atmospheres containing 21%, 2.5% or 0% O_2 (closed bars). The control cells were incubated under the same conditions but in the absence of cytokine (open bars). The data are expressed as the mean ± S.E.M. of $n = 5$ separate experiments each performed in triplicate. Under each O_2 condition tested, the addition of GM-CSF caused a significant inhibition of apoptosis ($P < 0.05$).

gen peroxide (0.1–5 mM) induced a concentration-dependent increase in the rate of apoptosis observed at 8 hrs (Fig. 4a). Cell viability as judged by Trypan-blue dye exclusion was not affected by hydrogen peroxide treatments below 5 mM (half-life of the hydrogen peroxide in this system was 2.25 h as determined by the reduction of absorbance at 240 nm). In view of the potent antioxidant capacity of serum (in our donors equivalent to a Trolox concentration of 1.08–1.12 mM [43], data not shown), the serum concentration in the Iscove's DMEM these experiments was reduced from 10% to 2%. The ability of 1 mM hydrogen peroxide to increase the number of cells undergoing apoptosis could be reversed by the addition of the antioxidant Trolox (10 mM), a soluble vitamin E analogue (Fig. 4b). This suggests that a potential pathway exists for oxidants to induce neutrophil apoptosis. However, it is important to note that activation of the respiratory burst by agents such as fMLP and IL-8 does not increase the rate of neutrophil apoptosis [13]. This, together with the observation that only an extremely high, supraphysiological concentration of hydrogen peroxide increases neutrophil apoptosis, and the inability of SOD, methionine and Trolox to mimic the effect of withdrawal of oxygen (Fig. 4b), implies that generation of superoxide anion, hypochlorous and peroxyl radicals may not be a major regulator of neutrophil apoptosis in vivo.

In conclusion, hypoxia caused a profound inhibition of neutrophil apoptosis in vitro. This effect is directly opposite to the pro-apoptotic effect of hypoxia observed in a number of cell-lines [20–21,28–30]. The ability of hypoxia to prolong neutrophil survival is additive to that induced by GM-CSF, is not associated with *bcl*-2 expression, and is not mimicked by exogenous SOD, methionine or Trolox. However, apoptosis was inhibited by catalase, but the exact mechanism underlying this effect and the potential role of hydroxyl radicals and hydrogen peroxide require further investigation. These data also imply that examination of apoptosis under routine hyperoxic (21% O_2) culture conditions may alter the susceptibility of cells to apoptosis and not accurately reflect the O_2 environment in vivo. In addition, neutrophils may remain alive and viable for considerably longer periods when recruited to an inflammatory

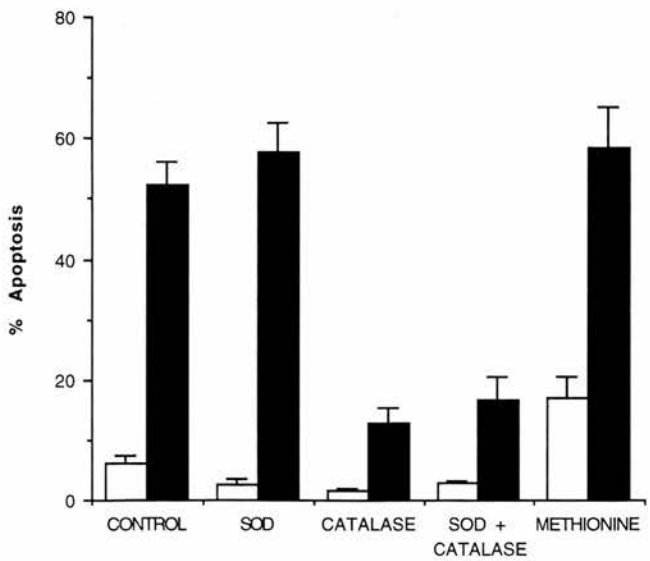

Fig. 3. Effect of SOD, catalase and methionine on neutrophil apoptosis. Neutrophils were cultured for 6 h (open bars) or 20 h (closed bars) in the presence of 200 μg/ml SOD, 250 μg/ml catalase, 200 μg/ml SOD and 250 μg/ml catalase, or 5 mM methionine. Control, untreated neutrophils were also prepared. Apoptosis was assessed morphologically. Data represent mean ± S.E.M. of $n = 3$ separate experiments, each performed in triplicate.

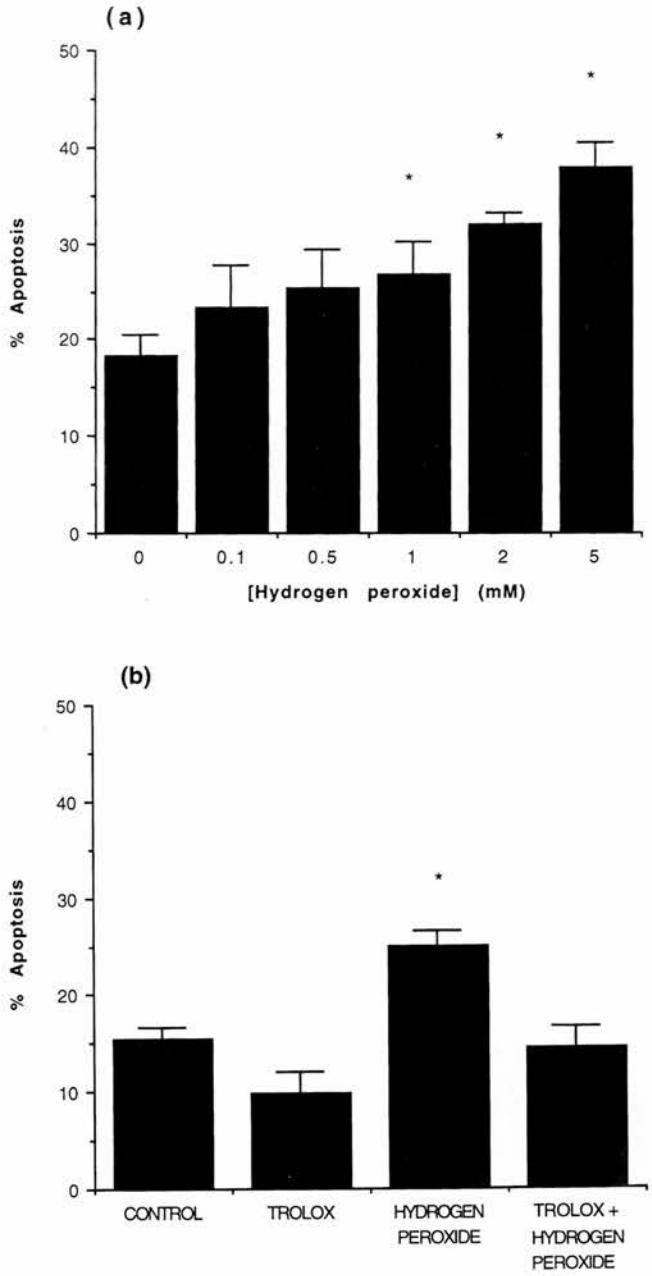

Fig. 4. Effects of hydrogen peroxide and Trolox on neutrophil apoptosis. Neutrophils were incubated for 8 h in the presence or absence of (a) increasing concentrations of hydrogen peroxide or (b) 10 mM Trolox ± 1 mM hydrogen peroxide. Apoptosis was assessed morphologically. Both sets of data represent mean ± S.E.M. of $n = 3$ separate experiments, each performed in triplicate. The rate of neutrophil apoptosis in response to 1, 2 and 5 mM hydrogen peroxide was significantly different from the control apoptosis ($^*P < 0.05$). In (b) the rate of apoptosis when cells were treated with both Trolox and hydrogen peroxide was significantly less than the rate of apoptosis when treated with hydrogen peroxide alone ($^*P < 0.005$); there was no significant difference between the rates of apoptosis of the control cells, Trolox treated cells and Trolox and hydrogen peroxide treated cells.

focus where there is local, and often systemic, hypoxia [23,24]. The effect on neutrophil survival, and hence delay in their removal, could potentially exacerbate the disease state and result in additional neutrophil-mediated tissue destruction. This finding may also have the practical consequence of stimulating research into the role of hypoxic conditions for prolonged granulocyte storage prior to transfusion.

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